

nature

3 April 1980

Chemical arms: a sea of troubles

RECENTLY, the worldwide press has reported on how rumours of Soviet chemical warfare in Afghanistan have led to increased support in the US for the resumption of manufacture of chemical weapons. Europe should take note of these reports, for if US chemical weapons production does resume it will almost certainly have been endorsed beforehand by at least one European government. Prior experience suggests that such endorsement will not receive prior parliamentary debate.

For several years European members of NATO have been under pressure from the United States to reconsider their positions on chemical weapons. Existing NATO policy is that chemical weapons are to be used only for retaliation in kind within the prevailing 'flexible response' defence strategy. US officials believe that this policy requires NATO to integrate a retaliatory chemical capability much more closely into its military planning and posture than it has so far done. France and the US are alone within the alliance in possessing significant stocks of poison gas, and, although certain other member-states, such as Britain, keep themselves informed about how to use chemical weapons, the remainder do not. West Germany, in particular, has long committed itself against acquiring its own, and has declared that it will not seek access to anyone else's.

The prevalent European view has been that the threat of escalation (including nuclear weapons release) implicit in the flexible response strategy, coupled with the antichemical protection of NATO forces, provides sufficient safeguard against Soviet resort to chemical warfare. Antichemical protection is currently being upgraded, and is capable of negating the mass-destructiveness of chemical weapons on the battlefield, albeit at some cost to fighting efficiency. French and American chemical weapons are seen as a useful extra precaution to the extent that they can act as an additional deterrent — a belief which may or may not be sound.

Suggestions that the NATO chemical retaliatory capability should be expanded have been opposed on several grounds. First, such an expansion could be interpreted as a sign of diminished resolve to use nuclear weapons. Increased deterrence of chemical warfare would then have been bought at cost of reduced overall deterrence. Secondly, there would be greater returns for European security from investment in improved conventional capabilities. In addition, a new chemical armament drive would compromise the current negotiations on chemical disarmament, which in theory offer the best safeguard against chemical attack.

While many Americans appreciate such arguments, some maintain that the present state of nuclear parity now thought to exist between the US and the USSR, together with the apparent Soviet interest in chemical weapons, strengthens the requirement for a special chemical-warfare deterrent. For reasons that are not entirely clear, they believe that their existing special deterrent — in the form of the one to two weeks' supply of US chemical munitions now on hand in West Germany, replenishable from much greater American stockpiles — is insufficient. Hence the plans for a new chemical weapons factory with a projected expenditure, so it is said in the American press, of \$1,300 millions, on an output of nerve gas artillery shells, bombs and warheads.

Such a programme can make sense only if Europeans undertake, at the very least, to accept the new weapons on their soil. This is the endorsement which potential host countries will be asked, once again, to give.

There are few public signs of the way European opinion may go on poison gas. The Afghan allegations, however unsubstantiated, could be influential, succeeding as they do a whole series of others that the USSR will use chemical and even biological warfare whenever it is militarily expedient, regardless of treaty

constraints. Reports have alleged Soviet backing for Vietnamese chemical warfare in Laos and Kampuchea, expansion of Soviet chemical-weapons stocks, and Soviet violation of the 1972 Biological Weapons Disarmament Convention. Yet, as a recently published review of these different charges shows in some detail*, no solid evidence has been disclosed for any of them.

Should the allegations be true, the implications for the West would be serious. The problem is that uncertainty about them extends to government — a symptom of the generally poor state of western chemical warfare intelligence. Because there is little hard information, these reports are in fact rooted in worst-case assumptions. Capabilities are estimated by equating them with the requirements for chemical weapons set by Soviet military doctrine on chemical warfare which, though not known with any precision, is assumed to teach maximum exploitation of all battlefield target effects available from toxic chemicals. The stockpile tonnage estimates quoted recently in the US press (*Nature* 13 March, 1980) are no exception. Their belittling of American capabilities is also commonplace, compounded in this instance by a confusion of chemical weapons with poison gas: it is the latter, not the former, of which the Americans have 42,000 tons — a supply sufficient to fill up to half a million tons of chemical munitions.

Absurdly, such estimates of Soviet stocks are frequently quoted by military analysts and journalists as evidence of the intentions from which, in fact, they were deduced. Because the Soviets have an enormous supply of chemical weapons, such comment goes, they must be planning for protracted chemical warfare of their own initiation. Yet such hard quantitative information as there is, drawn mostly from sightings of Soviet depots in forward areas believed to contain chemical weapons, is equally consistent with a retaliation-only posture, similar to NATO's.

In the historical record of US and Soviet chemical warfare preparedness since World War II, there is clear evidence that the programmes of one side have driven, at least in part, those of the other. The US stopped adding to its stocks of chemical weapons in 1969; the USSR, a year or two later. It would be highly dangerous if a cycle in the opposite direction were now to be set in motion again. The chemical arms limitation talks in Geneva, which are proceeding in both a US-Soviet working group and within the 40-nation UN Committee on Disarmament, provide what is probably the only available channel of communication for resolving such uncertainties. The first priority for the West in its policy-making on chemical warfare must therefore be to keep this channel open.

Over the years the negotiations have advanced to the point where they are now turning on intricate questions of the controls that must be placed on national chemical industries, and on particular military facilities, in the interests of adequate treaty verification. These raise issues of such delicacy that negotiation can proceed only if there is mutual confidence.

If the Soviets are innocent of the chemical and biological warfare allegations against them, a new western chemical armament drive cannot fail to deepen mistrust and suspicion. Although a go-ahead for the US binary nerve-gas plans could strengthen the position from which the West is negotiating, it could at the same time destroy the basis of negotiation. While Europeans may not be able to do very much to promote chemical arms control, they may soon be in a position to kill it. □

**Chemical and Biological Warfare: Analysis of Recent Reports concerning the Soviet Union and Vietnam, March 1980, pp42. Available as ADIU Occasional Paper no 1, from the Armament & Disarmament Information Unit, University of Sussex, Brighton BN1 9RF, UK price £1.50 plus postage.*



United States

Inventorship dispute stalls DNA patent application

Is scientific history being rewritten to make research results more patentable? **David Dickson** reports

A PATENT application covering some of the basic techniques of genetic engineering is being held up by the US Patent Office in a dispute over whether the applicants can legally claim to be the sole inventors. The patent request has been filed jointly by Stanford University and the University of California, San Francisco, (UCSF) on behalf of Dr Stanley Cohen and Dr Herbert Boyer. It covers techniques developed at the two institutions in the early 1970s, which demonstrated for the first time the feasibility of replicating biologically-functional foreign genes introduced into a living organism.

Central to the patent application, first filed in 1974, is a paper published in the *Proceedings of the National Academy of Sciences* (70, 3240, 1973) in the preceding year, which describes the successful construction and replication of a plasmid capable of transferring antibiotic resistance into *Escherichia coli*.

In addition to Cohen and Boyer, the paper names two other authors Dr Annie Chang of Stanford and Dr Robert Helling, Associate Professor of Botany at the University of Michigan. According to the rules of the Patent Office, joint authorship means the two are considered co-inventors of the processes described.

Stanford and UCSF argue that although the research described in the paper and the patent application was built on techniques developed elsewhere, it was Cohen and Boyer who provided the creative input necessary to demonstrate their potential. They did this by conceiving and demonstrating how the techniques could be combined to create functional recombinant plasmids.

But Helling, who is not named in the patent application, disputes that his role was marginal. He has refused to sign a disclaimer, required by the Patent Office before it approves the application, agreeing that he was not an inventor of the processes described.

"I felt that we were all equal in this, and do not want to sign a letter saying that I was just another laboratory worker", he told *Nature* last week. "I was part and parcel of the whole thing; I don't feel that I should sign something that I do not believe is true."

Helling is not the only scientist who has refused to sign (although his refusal has been the most disruptive). Dr John Morrow, of Johns Hopkins University, the first author on a 1974 *PNAS* paper cited by



Cohen: forgoing royalties

Cohen and Boyer (to show how a bacterial plasmid could be used to reproduce *Xenopus* DNA), complains in particular about the secrecy which has covered the application. "I am not prepared to sign a disclaimer to a patent application that I have not seen," he says.

And there may be further disputes in store. In addition to the process patent, Stanford and UCSF are also asking protection for the plasmid developed, arguing that it was the development of this particular pSC101 plasmid that made the success of the techniques possible.

Meanwhile, patents are being granted on the processes used. Dr Roy Curtiss, of the University of Alabama, has recently been granted a patent for the techniques used to create the disabled $\chi 1776$ strain of *E. coli* (for which both process and product patents have already been issued in the UK).

The issue has raised at least two separate arguments. The first is on the morality of granting private licensing rights to the results of research carried out on public funds. As this is currently accepted practice, most scientists feel that Stanford and UCSF are justified in seeking to benefit from research which the two institutions pioneered — particularly since the two universities stress that the patents would remain freely available to anyone who wanted to use the techniques for research purposes. And both Cohen and Boyer have agreed that royalty proceeds should go to

support research at the universities, and that neither will benefit directly.

A further difficulty arises from the clash between scientific conventions of co-authorship, and the legal implications of these conventions when it comes to claims of inventorship. The latter is a legal definition which courts have ruled is enshrined in the Constitution, but is seldom considered by scientists in determining whose name should go on a research paper. Patents can be granted for techniques whose details have been published less than a year previously, but where such prior publication has taken place, all those listed as co-authors are treated as *prima facie* co-inventors.

In the Stanford/UCSF case, all agree that the development of recombinant DNA methodologies relied heavily on discoveries made at several institutions, for example on work on restriction enzymes at Johns Hopkins University and by Paul Berg's group at Stanford, or on the functioning of ligases at the National Institutes of Health.

As Cohen puts it: "Scientific advances such as the one we have been involved in are in fact the result of multiple discoveries carried out by many individuals over a long period of time". The difficulty lies in evaluating the significance of any particular contribution.

Patent attorneys for the two universities argue that Boyer and Cohen provided the "major inspiration and direction" for the experiments described in the *PNAS* paper, and they have told Helling that, unless he signs the disclaimer — or they can convince the patent examiner by other means that he has no legal claim as an inventor — then they will seek a court order instructing the issuing of a patent, and 'deposing' him as an inventor. But a full-scale legal dispute is not a prospect that anyone fancies.

Legally-defined constraints imposed by commercial considerations are proving a problem for scientists concerned to retain the integrity of the research process.

"It is difficult to demand high standards of your students when you see some of the things that are going on", says Dr Mark Ptashne of Harvard University, who claims that colleagues are beginning to write papers and cite references. "In such a way that their patent applications are valid".

Dr Zsolt Harsanyi, who is currently heading a major study of the implications of recombinant DNA technology for the office of Technology Assessment, confirms that "in the past couple of months" scientists have begun to talk about the potential for rewriting scientific history as a "serious problem".

Some are now using this as an argument against permitting the patenting of research results at all. Others suggest that fields such as electronics and chemistry have faced such conflicts for years, and have learned to live with the consequences.

In the case of molecular biology, the learning process does not look like being a comfortable one. □

Third World science fund: promises unfulfilled

THIRTY-five countries last week pledged a total of \$36 million to a new fund being set up by the United Nations Development Programme to assist developing countries build up their scientific and technological capabilities.

The fund was agreed as an interim measure at last summer's United Nations Conference on Science and Technology for Development, held in Vienna. UNDP officials estimate that extra commitments already made increase the amount pledged to \$45.7 million; a number of other countries are expected to make additional contributions over the next few months. However, the figure reached is far short of the \$250 million which delegates to the Vienna conference, agreed should be a minimum target for the fund (contributions to which would be voluntary).

The developing countries had proposed a more ambitious financing system at Vienna, based on automatic contributions and aimed at targets of \$2 billion by 1985 and \$4 billion by 1990. But the developed countries put forward the proposal for the interim fund as an alternative. This fund is initially designed to operate for the two years 1980-81: during this period, a group of specialists will meet to decide whether the more ambitious proposal is a feasible proposition, and if so how it should be carried out.

Meanwhile plans are already being drawn up by the UNDP for allocating the money contributed to the fund. It is meant to support the implementation of recommendations agreed at Vienna, such as promoting scientific cooperation between developing countries on a subregional and regional level, and seeking to strengthen their "endogenous scientific and technological capacities".

Last week's pledging conference in New York was attended by delegates from 78 countries. Almost all said that they supported the goals of the fund, although many indicated that their governments had not yet had time to agree on the exact size of contribution to make, while others claimed that short-term economic problems made it impossible to make a contribution now.

Several contributions were received from developing countries themselves. These admitted that the size of the payments they were able to make was small compared to the overall target, but emphasized that the contributions were intended primarily as a token of support for the fund. Thus Samoa pledged \$1000, Tanzania pledged 30,000 Tanzanian shillings (equivalent to \$3,614), and Lesotho pledged \$575.

Several of the 'middle income' developing countries agreed to contribute larger amounts. Nigeria, for example, promised

\$250,000, China agreed to contribute \$264,000, and Mexico has indicated that it is likely to make a similar contribution.

Predictably — although with some surprises — the major contributions to the new fund will come from the developed countries. Of the definite commitments made in New York, Italy will contribute over \$9 million in line with its efforts to increase substantially its aid to developing countries, the Netherlands and Sweden will each contribute \$5 million, and Norway, Switzerland and Austria — the host of last year's conference — \$2 million apiece.

Delegates to the pledging conference from both France and Germany indicated that, although administrative reasons had made it impossible so far to earmark specific sums for the new fund, both intended to make contributions of about \$5 million.

Of the other major industrial countries, Britain had made it clear at Vienna that cutbacks in foreign aid made it impossible to commit any new money to development projects (and indeed no British delegation turned up for the pledging conference). Japan said it is still thinking about whether to contribute; and Canada stated that its government had decided to channel all new aid for science and technology in developing countries through the International Development Research Center (IDRC).

The US contribution, initially expected to be considerable, has fallen victim both to general cutbacks in federal expenditure, and to political conflicts that have surrounded Congress's successful torpedoing of the administration-backed Institute for Scientific and Technological Cooperation (ISTC).

In Vienna, US delegates had talked informally of a contribution in the region

of \$50 million. By the Autumn, this had dropped to \$25 million, and when President Carter's budget request to Congress was delivered in January, it had fallen to \$15 million.

Now it looks as if the contribution will be even lower. The US delegation to last week's meeting stated that its contribution would be between \$10 million and \$15 million — and it is thought that the lower of the two is the more likely. In addition, the US attached two conditions to its contribution. Firstly, that it should not be more than 20% of the total fund; and secondly that "significant contributions are made by countries receiving large incomes from oil exports".

Such contributions are expected, but have not yet materialised. Saudi Arabia, announced that it would indeed provide support, but that the precise amount was yet to be decided by its government. And Venezuela, one of the Latin American countries which had been arguing most strongly for new financial mechanisms, surprised many of those present by stating that it, too, while prepared to support the fund, had not yet decided on the extent of its support.

Despite some disappointment that the initial total had not been higher, UNDP said that they were well satisfied with the results of the pledging conference, and are now finalising procedures for deciding how the money should be distributed in a way that supports the final recommendations of the Vienna conference.

In closing the conference, UNDP administrator Bradford Morse said that the contributions received so far meant that the interim fund would be able to operate on 1 May, and several grants are expected to be made soon after that date.

Mr Morse stressed that the contributions promised were only "a first step" towards the target agreed in Vienna, and that a further opportunity for contributions would be provided at a later date.

David Dickson

'Golden Fleece' recipient settles for \$10,000

A MICHIGAN scientist who sued Senator William Proxmire for libel after the senator had awarded a "Golden Fleece" award for the scientist's research on the behaviour of animals under stress has accepted an out-of-court settlement of \$10,000.

The scientist, Dr Ronald Hutchinson, who was previously director of research at the Kalamazoo, Michigan, State Mental Hospital, had received a grant of \$500,000 from the National Aeronautics and Space Administration to support his research. However the grant had been ridiculed by Senator Proxmire as a waste of public funds, stating that the research "should make the taxpayer's as well as (Dr Hutchinson's) monkeys grit their teeth". Last year the Supreme Court ruled that Senator Proxmire could not claim

parliamentary privilege, since the remarks were made not only during congressional debates, but also in press releases and during television appearances.

In making the payment last week, Mr Proxmire issued a statement admitting that some of the statements made about Dr Hutchinson were incorrect. For example, he retracted the claim that Dr Hutchinson's projects were extremely similar and perhaps duplicative, and also that Dr Hutchinson had made a fortune from his research with monkeys. Senator Proxmire and Dr Hutchinson have agreed, following the settlement, that "further litigation is unnecessary". However, an aide to the Senator pointed out that the Senator had not apologised for making the award — and intended to make further "Golden Fleece" awards in future to those whose research projects sounded particularly pointless. □

Sweden

Limited nuclear programme favoured

In the referendum on nuclear power held on 23 March, 58% of Swedes voted for the completion of the present 12 reactor programme, but only 18.7% gave their approval to anything beyond that.

The referendum was a political exercise from start to finish (see *Nature* 13 March 1980, pages 117-8), and it came as no surprise that the combined forces of the two pro-nuclear options outweighed the support mustered by the single anti-nuclear one. Nevertheless, the success of the anti-nuclear movement should not be underestimated. Against the opposition of three of the four major parties, the trades union movement and industry, it managed — with far less money but far more personal engagement than its rivals — to gather nearly 40% of the voters to its side. The members of the anti-nuclear campaign, led by Lennart Daléus, are unanimous that their fight will go on, although the form of their future organization has yet to be decided.

On the pro-nuclear side, the softer option which recommended carrying out the planned 12-reactor programme but not building any more turned out to be marginally the most popular option of all, polling 39.3%. The more enthusiastically pro-nuclear option polled 18.7%. The fact that nearly 78% of the voters supported options which, with some credibility, demanded phasing out nuclear power, suggests that the population at large accepts nuclear power for a limited period while renewable energy sources are further developed, but by no means wholeheartedly embraces it as the energy system of the future.

Even if this is a reasonable interpretation of the attitude of most people, it remains to be seen whether the politicians will act on it. While they will be under intense pressure to increase funding for renewable energy sources, their immediate tasks will be to bring more reactors on-line. It seems certain that the Ringhals-3 and Forsmark-1 reactors will be quickly loaded: permission to load them has already been given but the actual loading was held up pending the referendum result. The State Power Board has also sought permission to load the Forsmark-2 and Ringhals-4 reactors, but as the reprocessing contract presently in force between the Swedish nuclear fuel supply company (SKBF) and the French firm Cogema does not provide for sufficient capacity to reprocess all the spent fuel from these two reactors, permission to run them will initially be limited to that time which will not produce more waste than the present contract could handle.

If, as the board hopes, Forsmark-2 were loaded in the spring of 1981, and Ringhals-4 in the spring of 1982, the present agreement would take care of

wastes produced from them both until the end of 1985. SKBF is currently trying to persuade Cogema to reprocess an extra 50 tons of spent fuel, which would cover the reactors until the end of 1986. Construction work will also go ahead on the depot for intermediary storage of spent fuel near the Oskarshamn reactors. According to a spokesman at the State Power Board, it seems unlikely that new reprocessing agreements will be concluded in the 1980s, so this depot will have to take care of spent fuel until new reprocessing agreements could be made in the longer term or until a decision were taken to store the waste directly, without reprocessing it.

Anti-nuclear Centre Party Prime Minister Torbjörn Fälldin has no intention of resigning, in spite of the fact that he had

staked all his prestige and moral strength behind the anti-nuclear campaign. His reaction to the result was to say that the people's desire for a maximum of 12 reactors must be respected. This has been seized on by his opponents as an opening shot in a campaign to do away with the twelfth reactor, Oskarshamn-3. The present government is a coalition of the Conservative, Liberal and Centre parties, and such a campaign would be carried on for purely political motives. It would be supported by some sections of the Liberal and Social Democratic parties, but staunchly opposed by the Conservatives' leader, Goesta Bohman, who backed the referendum's most pro-nuclear option. The coalition is undoubtedly in for some tough in-fighting.

Wendy Barnaby



Aldabra has the largest breeding colony of frigate birds (Fregata) in the western Indian Ocean.

Environment

Aldabra faces problems

ALDABRA, the atoll in the Indian Ocean that narrowly escaped becoming an air staging post 13 years ago, once more faces an uncertain future. On 31 March wardenship of the atoll and its ecological treasures passed from the Royal Society, which has maintained a programme of research and conservation since the first alarm, to the Seychelles Islands Foundation. This new public trust has members representing the Seychelles government, the Royal Society, the Smithsonian Institution and international organizations well attuned to the scientific value of an island virtually unscathed by human interference, but its task will not be easy. The foundation will need \$120,000 a year to maintain its research station and scientific programme.

The inhospitability of Aldabra has preserved it from most of the exploitation

suffered by other oceanic islands. The goats, cats, rats and mice introduced after 1880, when a small settlement began on the west island, have not wrought ecological havoc on the other three islands of the atoll. And so when the UK government announced in 1966 its intention to set up a military airfield on the atoll, ecologists realized that they were about to lose a unique ecosystem, where biogeographical and evolutionary theories could be tested.

The Royal Society became the focus for international concern, and mounted an expedition in 1967 to make an inventory of the terrestrial and marine features of the atoll before development began. But in the meantime the plans for the airfield were abandoned, and the society established a more permanent presence. A small research station was built, and in 1971 the society acquired the lease of the atoll, making possible a more comprehensive programme of research, which continued when Aldabra passed into the care of the Republic of Seychelles in 1976.

Since then almost 100 visitors have studied the geology, flora and fauna of Aldabra, making it one of the best known of all oceanic islands and coral atolls. Among the most spectacular of the fauna is the unique colony of about 150,000 giant tortoises, *Aldabrachelys gigantes* (a subspecies of *Geochelone*).

The future of this work is uncertain because the Seychelles Islands Foundation must rely on its own financial resources. In November 1979 it launched an appeal for at least a million dollars to provide an endowment, the income from which could be used to conserve Aldabra in perpetuity. So far more than \$300,000 has been raised, but that will not support the scientific presence much beyond the end of 1980.

Mary Lindley

The World Wildlife Fund, 29 Greville Street, London EC1 8AX, is a focus for the appeal in the UK.

PLANNED changes in Bulgaria's higher education system are designed to provide an annual crop of graduates whose training has been specially tailored to that year's economic needs. Final details of the plan have yet to be worked out, but it appears that at least some of those involved in the new scheme view it with misgivings.

According to Professor Angel Pisarev, Deputy Chairman of the State Committee for Higher Education, the need for the reform has its roots in the pre-war structure of Bulgarian education. At that time the country had no higher technical education whatsoever, and budding engineers had to go to Germany, France or Hungary for their training. Immediately after the war, it was decided to build up a network of polytechnics, giving a degree-equivalent training in such subjects as machine construction, electrotechnics, chemical engineering (including food technology), building and architecture. In all, Bulgaria now has 10 such polytechnics, five of them in Sofia.

Meanwhile, the pure sciences remained the province of the universities, (although a major change in 1972 "integrated" the University of Sofia and the Bulgarian Academy of Sciences, in a scheme by which the Academy took over part of the training of doctoral students, both bodies remaining autonomous).

Measures for implementing the new scheme were approved last year, and an

Eastern Europe

Higher education in Bulgaria

interim system was introduced this academic year to alter the content of lectures and "give more knowledge in the fundamental subjects and a greater practical knowledge at the end", plus an extra 4-5 months of practical education after the academic course but before graduation. The new scheme, to be introduced in 1981, is more radical.

First, the university or polytechnic course, only recently extended from four to five (officially 4½) years, in line with Soviet practice, will be extended to 5½ years. In the first two years, students will receive "fundamental preparation" in fairly generalized courses (physico-mathematical, chemical-biological, electrotechnical, etc.). At the end of that time, they will split up into "general specializations", on the basis of a document drawn up in consultation with factories, agroindustrial complexes and the like. The ministries concerned, having once ordered a certain number of students, will have to find them a job at the end of their

course. There will be no detailed assignment at this stage, since 15-20% of students fail to graduate.

Job assignment will come at the end of the fourth year. Prospective jobs will be posted on notice boards and research posts will be advertised at the same time. During the final year and a half, the student will receive his education partly at the university and partly at his future place of employment. (In the case of a future science teacher, he will do his pedagogics course at this stage.) Finally, after 5½ years the student will graduate and proceed to his appointed job.

According to advocates of the scheme, not only will the new method relieve the student of worries about future employment during their final years; it will also allow the unification of higher education throughout the country. In some years, Pisarev admitted, certain specialisations may be completely closed if no further specialists in those fields are deemed necessary.

Industry, said Pisarev, neither wants to nor can come to terms with the new system — a reaction which may not be unconnected with the fact that industry will have to bear some of the cost of laboratory work in universities and technical training. Bulgaria already has a contract research system involving some students. Now such research will be an obligatory part of the 'basic educational process'. **Vera Rich**

Interferon

Weizmann's interferon entries

THE latest entries for the great interferon race come from an international bevy of owners, headed by the Weizmann Institute in Israel. Not wishing to go nap on a single entry, the Weizmann has simultaneously backed three separate candidates in the hope that at least one will give them a share of the pay-off. Of the three, two involve cell culture techniques and the other embraces genetic engineering.

The genetic engineering project is under the scientific aegis of Professor Michael Revel of the Weizmann Institute and Professor Pierre Tiollais of the Pasteur Institute whose laboratories have considerable experience in interferon and recombinant DNA, respectively. Already their collaboration has resulted in *Escherichia coli* which are producing trace amounts of interferon, an achievement similar to that of molecular biologists in Zurich backed by Biogen (see *Nature*, 24 January 1980, p319) and, before them, Japanese scientists led by Professor Taniguchi at The Yeda Research and Development Company, which handles industrial processes arising out of the Weizmann's research, together with the Pasteur filed an Israeli patent application for the cloning of the human

interferon gene in November 1979.

Yeda is now collaborating with the Cetus Corporation in Berkeley, California in an attempt to improve the bacterial yields of interferon and to scale up the process.

Yeda is also involved in separate attempts to produce fibroblast and lymphoblastoid interferon by cell culture techniques. Professor Revel's team can already do that on a laboratory scale. To scale up production of lymphoblastoid interferon, an arrangement has been made to use the facilities of the Merieux Foundation in Lyons, France. For fibroblast interferon, Yeda and the Swiss Ares Company are building a plant in Israel.

For those who wish to wager a bet, it seems that the Weizmann's recombinant DNA entry is up with the leaders whereas the cell culture candidates are some way behind competitors such as Searle and Wellcome Research Laboratories. There are, however, many dark horses and undeclared entries to contend with. □

United Kingdom

Science budget steady

A FOUR-year period of relative stability for overall science funding is promised by the

UK Government in its White Paper on Expenditure Plans until 1983-84. But in the division of the 1980-81 science budget the Science Research Council have, on paper at least, received only 52.5% compared with 55% a year ago.

The money available in the 1980-81 science budget will be £383m. It is planned that the budget will be the same in real terms next year and 1% higher in 1982-3 and 1983-4. In announcing this, the White Paper says "The Government attach importance to the support of basic science, in which the United Kingdom excels, as an investment in the country's industrial and intellectual future." Whether or not that sentiment tallies with a four-year static science budget has to be judged in the context of a 9% reduction in expenditure on education planned over the same time.

Of the £383m science budget for 1980-81, the Agricultural Research Council will get £35m, the Medical Research Council £71.8, the Natural Environment Research Council £45m, and the Science Research Council £201.1m with the rest being divided among the Social Science Research Council, the British Museum, Natural History and the Royal Society. Compared to its share of the total a year ago, the Science Research Council appears to have lost out to the Medical Research Council (+1.5%) and the Natural Environment Research Council (+0.7%).

Peter Newmark

NEWS IN BRIEF

No change for US nuclear export licensing

UNDER heavy pressure from the US Congress, President Carter has backed off proposals to remove responsibility for issuing licenses for the export of nuclear fuels and technology from the Nuclear Regulatory Commission (*Nature*, March 20, page 201).

Several members of the administration, including Mr Gerard Smith, Assistant Secretary of State for non-proliferation matters, had lobbied strongly for the change, arguing that the NRC should not be given foreign policy responsibilities. The move was supported by the nuclear industry, which has recently suffered from a poor export performance. However it was firmly resisted by Congress, which argues that in allocating such responsibilities to the NRC under the Nuclear Non-proliferation Act of 1978, it had sought specifically to remove evaluations of the adequacy of safeguards from direct policy considerations. Following President Carter's rejection of the proposed change, an administration official was quoted as saying that the President did not consider it to be "the right time to raise the issue before Congress".

Photocopying company stops multiple copying

THE Gnomon Corporation, a photocopying corporation with branches in eight eastern university towns, including Cambridge (Massachusetts) and New Haven, signed a legal undertaking last week that it would not make multiple copies of portions of any printed material, even if the material does not carry a copyright notice. The company agreed to make the declaration following charges that had been brought against it by eight leading textbook publishers, and backed up by the Association of American Publishers, that material from their publications was being copied without permission, in violation of the revised copyright law of 1978.

The publishers presented evidence against the Gnomon Corporation which they claimed was typical of a huge volume of illegal copying. Each piece of evidence involved the photocopying of at least an entire chapter or complete journal, and in many cases a third to half of a book.

According to the vice-president of the publishers' association, most of the photocopying was of 20-30 copies for university lecturers. The eight publishers filing the suit included Prentice-Hall, John Wiley and Sons, and Princeton University Press.

Gnomon has also accepted responsibility for photocopying done on coin-operated self-service equipment in its stores. The

president of the Corporation, Mr Adam Carley, has said that parts of the agreement are highly objectionable. He considers that his company's copying practices were legal "but we couldn't afford the legal fees to fight the suit".

New committee on recombinant DNA research

AN interdepartmental committee has been established in the US to study the possible health implications of the industrial applications of recombinant DNA research, prompted by the concern of Dr Eula Bingham, Assistant Secretary of Labour responsible for occupational safety and health.

The committee is a subcommittee of the interagency committee on recombinant DNA research, and will be chaired by Dr Gilbert Omenn, associate director of the Office of Science and Technology Policy. The executive secretary will be Dr Bernard Talbot of the National Institutes of Health.

The new committee, which is holding its first meeting next week to decide on a plan of work, has been set three tasks by the interagency committee. First to review the industrial applications of recombinant DNA research. Secondly to assess the need for providing educational information to workers about the techniques they will be coming in contact with. And thirdly to determine criteria for examining the question of potential hazards.

Five-year anti-nuclear campaign launched in UK

FRIENDS of the Earth has launched a £1 million, five-year campaign against the Conservative Government's plan to introduce pressurized water reactors into the UK at the rate of one each year starting in 1982. The campaign aims to turn nuclear power into a major issue of the 1984 general election. It plans to do this through a five-year strategy consisting of increasing public awareness through demonstrations, local elections, and summer schools on nuclear power in the first year; continuing education and initial fund-raising in the second and third years and community organizing and mobilization in the fourth year. The election campaign in the fifth year will support candidates who declare themselves to be against nuclear power.

According to Czech Conroy, FOE campaign director, the programme will emphasize three policy issues — safety, the record of the nuclear industry, and the cost of nuclear power — and three political issues — governmental secrecy on nuclear power issues, the use of taxpayers' funds for pro-nuclear campaigns and the morality of risking nuclear power development without adequate safety and waste disposal facilities.

Research reactor for Bangladesh

THE Bangladesh Atomic Energy Commission recently signed a contract with the US company General Atomic for the supply of a 3-MW TRIGA Mark-II pulsed research reactor. The TRIGA (Training Research and Isotope Production Reactor by General Atomic) is a water cooled, graphite reflected reactor suitable for research training and isotope production. It has a steady state power level of 3 MW and a peak power output of 1,200 MW. The reactor will be installed at the Bangladesh Atomic Energy Commission's research establishment at Savar, about 50 kilometres north of Dacca.

M. Kabir

Givaudan negotiates out-of-court over Séveso

THE Givaudan Company, a subsidiary of Hoffman-La Roche, is negotiating with the Italian government and regional authorities for payment for damages caused by the uncontrolled release of dioxin from its ICMESA chemical plant in Séveso on 10 July 1976. If the Commission of Parliament for Lombardy approves the settlement, Givaudan will pay £24.3 million to the Italian state and the Lombardy regional authority for their expenses incurred in public health, social assistance, damage to the environment, and loss of local industry and trade. Givaudan will also complete at its own expense the decontamination work currently underway.

AUT fights London layoffs

In a letter to Lord Annan, the Vice-Chancellor of the University of London, the Association of University Teacher criticizes the Flowers report on the reorganization of medical education in London (*Nature*, 6 March, page 5) for its potential to make teaching and research staff "the victims of proposals to cure the alleged defects of a system for whose construction they bear no responsibility".

According to John Akker of the AUT, the Flowers report does not say how its proposal to save £3 million from "rationalization" of staff (out of a university budget of £200 million) should be put into effect. The AUT also criticizes the report for not first estimating whether or not savings could be made from sales of buildings and saved maintenance costs and also for not including the cost of redundancy payments and rehousing of staff in expanded facilities at non-threatened institutions.

East-West fencing at Geneva

The outcome of the recent review of biological weapons control does not bode well for other arms control negotiations, writes **Julian Perry Robinson**

THE 1972 Biological and Toxin Weapons Convention (BWC) represents the only real disarmament measure agreed since the World War II peace treaties because it is the only one that has required the destruction of existing stocks of weapons. But, as a model for further agreements, it is weakened by flimsy arrangements for verification. These were the subject of fierce controversy at a conference to review the convention, held in Geneva at the end of last month.

At the one extreme was the Swedish delegation seeking amendment of the treaty to provide for an expert consultative committee under the UN Secretary-General, empowered to investigate complaints of violation. At the other extreme was the Soviet delegation, arguing that since no one had yet raised a complaint under the procedures which the treaty already provided, there was no need to elaborate additional procedures. Between the two was the UK.

Two thirds of the way through the conference, however, there was a turn of events which paradoxically strengthened the position of the USSR. The US State Department suddenly used one of its daily press briefings in Washington to declare itself suspicious of Soviet compliance with the BWC, citing an unexplained outbreak of infectious disease in Sverdlovsk eleven months previously. In various different forms since October, this outbreak has been reported in the British and West German press, with speculation that an accident had occurred in a clandestine biological weapons facility (*Nature*, 27 March, page 294).

The abruptness of the US announcement, and its failure to display any very solid substantiation, strongly suggested that the Americans had launched a wrecking operation, driven by growing anti-arms control sentiments in Washington, and perhaps by the pro-chemical weapons lobby. The drafting party which was then putting together the bones of the final declaration of the conference felt compelled to avoid language which suggested that the BWC had been fully observed, but the US delegation eventually let it be known that it would not raise the matter at the conference until the final plenary session. Since the text of the final declaration should by then have been adopted, this implied that the US delegation was, after all, going to maintain its initial stance of preserving the BWC against damaging aspersion.

The Sverdlovsk allegation very much affected the content of the final declaration

on the thorny issue of the consultative committee. On the one hand it illustrated most graphically the need for some form of international verification procedure. On the other, it suggested that the USSR would be the subject of the first complaint to be brought before the committee, and few states were happy to contemplate the political furore that would ensue, and the attendant threat to the BWC's continuation. The outcome was ambiguous talk about the possibility of a "consultative meeting . . . at expert level" being convened under the verification article of the treaty, without details about who would convene it or what it was supposed to do. Its only merit is that it leaves the question open for further consideration "at an appropriate time". The Soviet delegation had argued that a review conference was not a proper place for amending a treaty — a principle which many delegations subscribed to, including those worried about verification (presumably because of fear of setting a precedent for the 1968 Non-Proliferation Treaty review in August this year).

Some delegations appeared to have taken note of a submission made by the Pugwash movement, which in one of its East-West conferences of senior scientists back in 1959 had set the ball rolling towards international agreement on the BWC. Part of its submission was that highly toxic substances of military potential might well become accessible via certain of the new biotechnologies that are beginning to attract heavy investment, and that if this were to happen it would not be clear whether the substances would be treated as toxins within the meaning of the BWC.

The three co-depositaries of the convention — the US, the USSR and the UK — were reluctant to concede that there might ever be any problems over the scope of the treaty, for this might undermine confidence in it; and they produced an anodyne joint paper on scientific developments since 1972, including DNA hybridization techniques, which concluded that nothing had happened which necessitated any clarification of scope. The conference did not take serious issue with this conclusion. But it decided, largely on the insistence of Sweden, to hold a further review conference sometime between 1985 and 1990.

There are certainly several loose ends hanging out of the BWC which might cause problems as long as there is no analogous convention on chemical weapons to tie them up. This is recognised in Article IX of the BWC, which requires party states to

"continue negotiations in good faith" on chemical weapons. When the operation of this article came under review, many of the delegations strongly criticised the two superpowers for the apparent absence of major progress in their secret bilateral negotiations on the subject (the eleventh round of which was then in session down the street), and for their opposition to the opening of complementary negotiations within the multilateral UN Committee on Disarmament (the CD). Chemical weapons reached the CD agenda soon after the BWC review conference had begun, which seems to have hastened a compromise on the complementary negotiations issue. But apart from the Americans, no one seems at all happy with the compromise, which calls for the convening of an *ad hoc* working group of the CD to "define, through substantive examination, issues to be dealt with in the negotiation on a chemical weapons convention". The primacy of the bilateral negotiations is thus preserved for the foreseeable future, so that the prospects for worldwide prohibition of chemical weapons will remain dependent on the caprices of the superpowers.

The BWC review showed very clearly that the superpowers are in no mood to accommodate one another on chemical or any other arms-control matter, despite growing international tension. The Americans seem set on a policy of brinkmanship threatening (as with the current calls from Washington for an expansion of NATO chemical-warfare capabilities (see page 286)), to destroy the basis of negotiation. Soviet reactions (like their refusal to accept the modest degree of international verification inherent in the BWC consultative committee proposals) are commonly seen in the West as intransigence on basic issues. Neither has the USSR displayed any understanding of the roots of western mistrust. It did not respond to the suggestion that states should voluntarily declare whether or not they had formerly possessed biological weapons.

Many of the participants to the conference regarded the affair as a dry run for the second NPT review conference in August. This attitude no doubt stemmed from the fact that biological weapons make little sense militarily and have never been developed to their full mass-destructive potential. But the chief significance of the meeting lay rather in the influence it might have on other arms control negotiations.

In private conversation about the Sverdlovsk allegation, a Soviet representative doubted whether there could now be any progress in the CD on chemical weapons or anything else at all important this year. Perhaps he was speaking in the heat of the moment — but the present climate does not favour optimism. □

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CORRESPONDENCE

Chinese Scientific American

SIR, — In the second of his three recent articles on the development of science and technology in China (*Nature* 7 February, page 516) Tong B. Tang makes the statement, based on his visit to China at the end of last year, that "There are no cover-to-cover translations into Chinese" of foreign scientific journals. It may interest your readers to know that this statement was not true then and is not now. *Ke Xue*, the Chinese-language edition of *Scientific American*, began regular publication in China with its January 1979 issue, lagging the parent English-language edition by 14 months. Beginning with the January 1980 issue *Ke Xue* will lag the parent edition by only four months. The paid monthly circulation of this "cover-to-cover translation into Chinese" of a foreign scientific journal is currently 25,000.

GERARD PIEL

Scientific American, New York, US

How safe are safety cabinets?

SIR, — There has been much concern about the performance and testing of microbiological safety cabinets built before the publication of the new British Standard (BS 5726) (*Nature*, 278, 29 March, 1979, p.384). This concern is now being extended to cabinets built after the introduction of the standard and claiming conformity with it. Many of the problems are centred on the Class II Safety Cabinet which is designed to provide protection both to the work and the worker. In terms of safety, the two most important features of such cabinets are:

- that pathogenic aerosols are adequately contained by the inflowing air-streams; and
- the reliability of the filtration system is assured.

The BS allows that once the performance of a prototype cabinet has been validated, the subsequent production versions may be assumed to have satisfactory performance, provided that they have the same physical dimensions and that the air-flows do not differ by more than $\pm 10\%$ from those of the prototype. We do not believe that this principle is in practice adequate to provide safe working conditions, particularly in open-fronted Class II cabinets.

We have undertaken trials to evaluate production versions of a number of these cabinets and to correlate different methods of determining operator protection factors. Early in these trials the disturbing fact emerged that several cabinets reputedly identical in design to prototype models which had performed satisfactorily, failed to provide the required operator protection. The monitoring instruments of the cabinets indicated safe working conditions, and it was only from the results of aerosol containment tests that faults were revealed.

Most of the defects like faults in fan motors and their controls, or defective filter seals could have been rectified on site. But, more important, we have found that relatively low air movements in laboratories can severely affect containment; the approved design may not be adequate in such circumstances.

We urge most strongly that commissioning of new cabinets should include tests for containment, filter integrity and the quality of their seals; without these on-site tests there can be no guarantee that the cabinet will perform within specification.

Section six of the Health and Safety at

Work Act places an onus on suppliers, manufacturers and installers to provide cabinets suitable for the use for which they were designed and complying with the requirements of BS 5726. The customer should satisfy himself that these obligations have been met. Such tests must be included in the purchase price and not left as options for intending purchasers.

The DHHS operates an effective evaluation scheme for Class I cabinets, and there is clearly a need for a similar service for Class II types. This should cover all the requirements of the British Standard, but should in our view be supplemented by a test following installation on site. Agencies operating a rigorous approval scheme should be accredited by the British Standards Institution.

The results of the recent trials will be reported in detail in due course. One finding can be mentioned now: namely, that when tests using micro-organisms are unacceptable an alternative method making use of an aerosol of potassium iodide may be a suitable substitute.

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Synergism v. cost benefit

SIR, — In a disturbingly high proportion of experiments on mixtures for pest and disease control, decisions are based on questionable criteria. Many biologists appear to have become convinced that the ultimate goal is synergism. In screening candidate mixtures for further study a mixture that is not considered synergistic is sometimes abandoned.

Certainly, it is desirable to gain understanding of the mechanism of joint action, but often the need is only to assess the net observable effect of applying one mixture or another. The purpose is usually to minimize an overall cost, where the cost or loss function may include not only the cost of materials, equipment and labour, but also the losses in terms of environmental and health hazards, and damage to non-target organisms. On this basis, even mixtures with some degree of antagonism may sometimes be worth considering.

The point at issue is illustrated in Fig. 1, which shows contours of equal effect (isoboles) and of constant cost, plotted against rates of application of the components of a two-component mixture, assuming each component to be individually active. If the cost is linearly related to the amount of each component (Fig. 1a), a mixture can have no advantage over a single material unless the isobole at the required level of response is

sufficiently concave upward. The requirements for upward concavity are more severe if the cost curve is also concave upward (Fig. 1b), but if the cost curve is concave downward (Fig. 1c) even a mixture with a concave-downward isobole might be of practical value. Cost curves such as these can arise, for example, if either component has adverse effects on non-target organisms or on the environment, often the very reason for investigating a mixture.

All this may seem to be so obvious as to need no stating, but mathematically-inclined scientists might be surprised to discover the extent of the primary emphasis on synergism, and the relatively slight or belated consideration of cost-benefits.

Even if demonstration of synergism is considered relevant, there are varied concepts of what constitutes synergism, except in the case where only one component of a mixture is active on its own. I have elsewhere reviewed some of the confusion that exists in one field of application, a confusion that has its roots in failure to realise the importance of defining an appropriate reference model to represent absence of synergism (and antagonism). For example, many weed scientists, using a method due to Colby, consider that any mixture is synergistic if the proportion surviving is less than the product of the proportions that survive the separate components. On this basis, a "mixture" of two aliquots of the same material would be judged synergistic.

Other biologists consider a mixture to be synergistic only if its isobole is concave-up. The models corresponding to absence of synergism by these two definitions are, in general, quite different. Either of them, or neither, may be biologically relevant. Occasionally both definitions are even treated as if synonymous. Another common misconception is exemplified by the "definition" in the *Herbicide Handbook*. "Complementary action of different chemicals such that the total effect is greater than the sum of the independent effects". On this basis, a mixture for which the percentage responses to the separate components sum to more than 100 would always be classified as antagonistic.

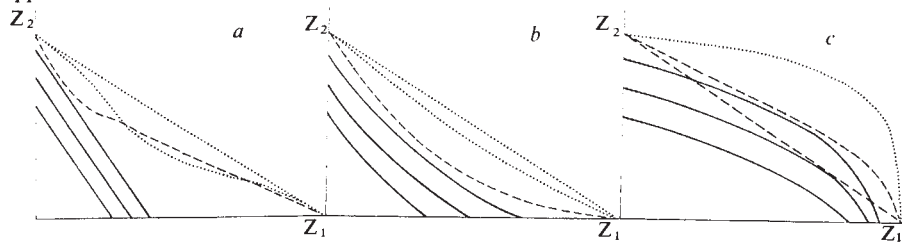
The problems of definition are neither easy to resolve nor to explain and demonstration of synergism in a fundamental sense may require specialized biochemical experimentation.

The aim of this communication is to plead for recognition of the real purpose of any study of joint action, and for appropriate assessment of the results. Cost curves may be difficult to establish in absolute terms, but even a rough idea of their forms could usefully influence the focus of screening and field trials of mixtures.

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Fig. 1 Curves of constant response (broken lines) and curves of constant cost (solid lines) for mixtures of two materials S_1 and S_2 at doses Z_1 and Z_2 respectively. The cost attributable to S_2 has been assumed to be proportional to Z_2 , so that in each diagram any two cost curves are at a constant vertical distance apart. Examples in which a mixture would be economically advantageous have isoboles shown by dashes (---); mixtures with dotted isoboles (....) could not cost less than S_2 applied alone.



NEWS AND VIEWS

Archaean stromatolites and the search for the earliest life

from Euan G. Nisbet

SEARCHING for the oldest traces of life on Earth is a difficult business — if the evidence for the existence of life is good, the rocks are usually not very old; if the rocks are very old the evidence for life is usually not convincing. In this issue of *Nature* Lowe (page 441) and Walter *et al.* (page 443) separately report two probable examples from the Pilbara, Western Australia, of $3.4\text{--}3.5 \times 10^9$ yr old stromatolites (sedimentary structures formed by algae or possibly other organisms). These may be the oldest macro-‘fossils’ yet found. These two reports and other recent work suggest that life began very early indeed in the Earth’s history.

To be convincing, a report of evidence for life in the Archaean (that period before 2.5×10^9 yr ago) must demonstrate both that the structures described are unambiguously biogenic and that the geochronological control is accurate. Cloud and Morrison (*Precamb. Res.* **9**, 81; 1979) distinguished three categories of evidence, discriminating between ‘permissive’, ‘presumptive’ and ‘compelling’ evidence for life. ‘Possible’, ‘probable’ and ‘definite’ (as far as any geological evidence is definite) would be another way of putting it. Very little of the evidence for life in the Archaean is ‘compelling’, but there is a considerable body of evidence in the ‘presumptive’ class. Careful geochronological work, in particular by O’Nions, Hamilton and coworkers, by Pidgeon and by Moorbath in various very old terrains is promoting the search for better and older evidence.

Palaeontology in the Archaean takes three main forms: the study of possible microfossils; the analysis of rocks for chemical traces of life; and the study of stromatolites. The identification of Archaean microfossils is often not wholly convincing (Muir *Publs. Geol. Dept., Univ. Western Australia*, **2**, 11; 1978). Microstructures which could be fossils have been reported from the Barberton area (3.5×10^9 yr) by Knoll and Barghoorn (*Science* **198**, 396; 1977) and from the Pilbara by Dunlop *et al.* (*Nature* **274**, 676; 1978), but in their review Cloud and Morrison concluded that the oldest structurally preserved microfossils for which there was conclusive evidence were 2.3×10^9 yr old (post-Archaean). The older examples from Barberton and the Pilbara are ‘possibles’, but the evidence is not conclusive. The second line of research, chemical analysis, includes both stable isotope work and the study of organic compounds in kerogen extracted from Archaean sedimentary rocks. Schidlowski *et al.* (*Geochim. cosmochim. Acta* **43**, 189; 1979) studied carbon isotopes in 3.7×10^9 yr old Isua metasediments, from West Greenland. Their evidence suggested that the terrestrial carbon cycle was virtually stabilized at least as early as 3.7×10^9 yr ago — the oldest ‘possible’ evidence for life on Earth. Sklarew and Nagy (*Proc. natn. Acad. Sci. U.S.A.* **76**, 10; 1979) have isolated possible remnants of biologically made carbohydrates from the Belingwe stromatolites, Zimbabwe Rhodesia (2.7×10^9 yr old). However, post-depositional contamination is a major problem in this research, especially when early Archaean samples are studied.

Archaean stromatolites are on a much larger scale — they can be ‘hit with a

hammer’, but in some cases it is difficult to distinguish between stromatolites and similar but abiogenic sedimentary structures. At best stromatolites offer ‘compelling’ evidence for the existence of life, since analogous modern structures are well-known and have been studied in detail (particularly at Shark Bay, Western Australia). Thus the evidence reported by Lowe and Walter *et al.* is of especial importance. At present the oldest compelling evidence for life on Earth comes from stromatolites about 2.7×10^9 yr old, especially from the Slave Province, Canada (Henderson *Can. J. Earth Sci.* **12**, 1619; 1975), from Steep Rock Lake, Canada (Jolliffe *Econ. Geol.* **50**, 373; 1955) and from the spectacular outcrops near Belingwe, (Bickle *et al. Earth planet. Sci. Lett.* **27**, 155; 1975, Martin *et al. Precamb. Res.* in the press). Slightly older, and not so well exposed, are the probable stromatolites (Mason & von Brunn *Nature*, **266**, 47; 1977) of the Pongola Supergroup, S. Africa ($2.9\text{--}3.0 \times 10^9$ yr old). Stromatolite-like structures also occur in the Barberton area, S. Africa, but they may be of inorganic origin. Thus these two new reports of 3.5×10^9 yr old stromatolites add very significantly to the evidence for the existence of life in the early Archaean.

Lowe describes internally laminated conical mounds, interpreted as stromatolites, in recrystallized evaporites which form part of the Warrawoona Group. Criteria for biogenicity cited by Lowe include morphological similarity with younger stromatolites and similarity of sedimentary environment with later examples. Lowe considers that forms similar to *Conophyton* occur in the Pilbara: if so, this very considerably extends the supposed age range of this type

of stromatolite since the only other Archaean example of *Conophyton* is at Belingwe (Martin *et al.* *Precamb. Res.* in the press). Walter *et al.* report a single nodular structure, also from the Warrawoona Group. They consider that this structure could not have formed abiogenically and thus identify it as a stromatolite.

How will these two reports be regarded by properly sceptical geologists? 'Probable' is the most likely response. The burden of proof is very great in material as old as this, but it is interesting that 'possible' microfossils of the same age have been reported from the same area by Dunlop *et al.* Further fieldwork may produce more definite evidence. Thus the oldest 'possible' evidence for life is that

from Isua (3.7×10^9 yr old); the oldest 'probable' evidence is from the Pilbara stromatolites reported in this issue of *Nature*; and the oldest 'compelling' evidence from the superb late Archaean stromatolites in Canada and near Belingwe. Nevertheless, the 'probable' evidence from the Pilbara, together with the possibility that the Isua rocks do record biological activity, suggest that life on Earth began very early indeed in the geological record. It is difficult to imagine that the surface of the Earth was hospitable to life before, say, $4.2\text{--}4.3 \times 10^9$ yr ago. Thus, if life did originate on Earth, the processes leading up to it (*Proverbs* 8.27) must have happened very quickly indeed. A few hundred million years is not long on a geological time scale. □

Fishing for oncogenes

from John Wyke

SOME 10 years ago the existence of particular genes which might be responsible for inducing cell neoplasia was first suggested. This 'oncogene hypothesis' proposed that the RNA tumour viruses owed their ability to transform cells to transforming genes (oncogenes) which they may have picked up at some time, perhaps from the host cell itself. In the past decade a wealth of evidence has partially validated this idea, for we now know that the transforming genes of many retroviruses contain sequences that are homologous to regions in the genome of normal cells, the virus presumably having acquired this genetic material by recombination between its genome and the host cell. These cellular homologues of viral oncogenes may themselves be oncogenic if expressed at inappropriately high levels (as may occur when their expression is controlled by a retroviral promoter) and neoplasia may simply be a matter of gene dosage. On the other hand they may become oncogenes only after mutation by physical or chemical means or during or after recombination with a retrovirus (there may or may not be an additional requirement for inappropriate expression). These putative cellular oncogenes clearly have a potential importance beyond the interests of tumour virology. We would like to know their function, how they are regulated and whether their expression is important in cancer induced by agents other than viruses, and for these reasons they are attracting increasing attention.

Two strategies for identifying and isolating cellular transforming genes have recently been reported. One uses the technique of DNA transfection in which

naked DNA from a donor cell is introduced into a recipient, inducing a phenotypic alteration (in the case of putative oncogenes the phenotype sought is morphological transformation). Two groups, both with experience in transfecting retroviral DNA, have applied donor DNA from normal and chemically transformed cells to recipient untransformed NIH 3T3 mouse cells. G.M. Cooper and colleagues (this issue of *Nature* page 422) reasoned that normal cells might contain unexpressed transforming genes which, if they integrate adjacent to an active promoter in a recipient cell, could be expressed. They found that high molecular weight DNA ($> 20 \times 10^6$) from both normal and chemically transformed avian and murine cells did not increase transformation of recipient cells above background levels but that fragmented DNA from the same donors, sonicated to molecular weights of $0.3\text{--}3 \times 10^6$, transformed recipients at a low level (0.003 transformants per μg DNA). They suggested that fragmentation may have separated potential transforming genes from linked cell regulators and these liberated oncogenes, in a few instances, integrate next to an active promoter in the recipient. This interpretation was supported by using DNA from the transformed recipient as a donor in further transfections, when it was found that the high molecular weight DNA was now a very efficient transformer (0.1–1.0 transformants per μg DNA). This indicated to Cooper *et al.* that the oncogene and its newly-acquired active promoter were now transfected in tandem.

These results provide *prima facie* evidence that normal cells contain potential transforming genes, but one slight caveat is the possibility that DNA from the original donors contained not

oncogenes but promoters or other DNA sequences which, by virtue of their integration, induced expression of transforming genes in the recipient NIH 3T3, a cell line and therefore not strictly a normal cell. A more surprising feature of this work is finding that the DNA from all chemically transformed and normal cells tested transforms recipients with equal efficiency, suggesting that the chemically-induced oncogene expression is transmitted poorly to recipients. The author's explanation is that the chemicals may have mutagenically inactivated a *trans*-acting repressor of a transforming gene; the oncogene will therefore never be expressed in a recipient in which this repressor is functional and the transformation is thus phenotypically recessive.

Somewhat different results have been obtained by R.A. Weinberg's group (Shih *et al.* *Proc. natn. Acad. Sci. U.S.A.* **76**, 5714; 1979) who transfected DNA from 15 chemically transformed cells to test the hypothesis that transformation was due to the mutagenic action of the chemicals. Although these workers apparently used high molecular weight DNA, they found that DNA from 5 of the clones transformed with high efficiency, equivalent to that observed by Cooper *et al.* only with DNA from transformed recipient cells (not surprisingly, secondary transfections by Shih *et al.* with DNA from transformed recipients did not further enhance transformation). DNA from the other transformed clones and controls showed no significant transformation, in line with the observations of Cooper's group.

It is difficult to compare the findings of these two groups, given the different premises with which they prefaced their studies and differences in materials and technique. Indeed, only one cell line, the methylcholanthrene-transformed BALB 3T3 mouse cell MC5-5, was common to both studies and this, in Cooper's hands, yielded poorly transforming DNA whereas for Shih *et al.* it was an efficient transformer. However, it does seem likely that Cooper *et al.* were studying cells similar to those which Weinberg's group regarded as irreproducible inducers of transformation (Shih *et al.* apparently never used low molecular weight DNA for transfection). Moreover, Shih *et al.* provide clear evidence for cell transformation resulting from a dominant mutation. Whether these mutations can be in either or both structural or regulatory regions remains to be determined and these findings must be reconciled with the evidence of Cooper *et al.* that genes from normal cells can also induce transformation. As both groups realise, elucidation of these interesting questions requires the application of molecular cloning techniques to provide large quantities of transforming cell DNA and Weinberg *et al.* (12th Miami Winter Symposium, 1980, in the press) have

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already embarked on this project.

The other strategy for picking up oncogenes uses the penchant of retroviruses themselves for acquiring cell sequences. Essentially, the idea is to passage a non-transforming virus through a cell which is transformed, and thus expressing cell oncogenes, reasoning that recombination between virus and oncogene can occur, giving rise to a transforming virus. This ploy should permit not only stable acquisition of the oncogene but also its efficient transmission in an expressed form, using the virus as vector, readily enabling studies on the oncogene product and its phenotypic consequences. Naturally, a number of groups over the past 10 years have tried to

capture oncogenes in this way, but with limited success. However, Rapp and Todaro (*Proc. natn. Acad. Sci. U.S.A.* 77, 624; 1980) now claim to have developed a method for generating *in vitro* new mouse retroviruses capable of inducing leukaemias, sarcomas or carcinomas. Although they have not yet purified and characterised their new isolates, their protocol looks promising.

These two experimental approaches together provide a rational way to identify and amplify cellular transforming genes and initial results are encouraging. Soon they may enable us to learn a great deal about the variety, structure and regulation of oncogenes, and how their products effect the cell phenotype. □

Reintroduction of genetically transformed bone marrow cells into mice

from Bob Williamson

MANY human hereditary diseases are caused by the malfunction of a single gene coding for a protein, of which the human haemoglobinopathies are the best characterised. Methods based on recombinant DNA techniques now allow fetuses with these disorders to be identified more reliably and easily. Antenatal diagnosis with the possibility of offering abortion is now a reality for several of the haemoglobinopathies. Another (or complementary) approach to the problem of these genetically simple diseases would be gene replacement, in which a normal copy of the deficient or abnormal gene is introduced into the affected tissue after birth. So far this approach remains firmly in the realms of speculation but recent developments in the introduction of genes into mammalian cells are bringing it nearer. The most recent report from M.J. Cline *et al.* at the University of California, Los Angeles (this issue of *Nature*, page 422) concerns the reconstitution of bone marrow in mice with normal bone marrow cells into which a drug-resistance gene has been introduced *in vitro* and the demonstration that cells which are expressing that gene can be selected by treatment *in vivo*.

The initial problems to be faced in developing methods of gene replacement are first, to introduce the required gene into the appropriate cell *in vitro* and to build into the process some way of easily recognising and selecting transformed cells in which the gene is functional. Second, an

effective population of treated cells has to be reintroduced and maintained.

Attempts to transform mammalian cells with fragments of DNA (transformation here means the introduction of DNA into a cell with a consequent change in its phenotype and does not refer to neoplastic transformation) come up against several difficulties. Mammalian cells will accept 'foreign' DNA although normally at very low frequency and there is some evidence that it may be integrated into their chromosomes. The low frequency of transformation is partly because techniques for introducing DNA into cells in culture are rudimentary, but also because only a small proportion of cells in a population appears 'competent' to accept DNA from solution. It is also difficult to find appropriate mutants of mammalian cells which allow selection for newly-introduced gene function.

One way of introducing DNA into mammalian cells, which provides both a selective marker and a higher rate of introduction, is to use modified versions of certain DNA viruses such as SV40 and polyoma as vectors. If the inserted gene is in the correct orientation and near to a functional viral promoter RNA is made from the inserted gene as well as from the virus and genes inserted into these vectors can be properly transcribed, processed and translated (see for example Mulligan *et al.* *Nature* 277, 108; 1979; Hamer & Leder *Nature* 281, 35; 1979 using β -globin DNA).

Another approach, taking the herpes virus as the starting material is to use the virus thymidine kinase (TK) gene as the selective marker for transformation of TK-deficient cell lines by genes linked to

fragments of virus DNA containing the TK gene. At first it was assumed that the required gene would have to be chemically linked to the TK gene but more recently it has emerged that the small number of 'competent' cells that accept the TK gene will at the same time accept other DNA fragments present in the culture medium (Wigler *et al.* *Cell*, 16, 777; 1979) Correct transcription and processing of rabbit globin genomic DNA linked to a cloned herpes TK gene and introduced into mouse cells, has been shown (Mantei *et al.* *Nature* 281, 40; 1979).

But there is a prejudice about using viruses, even small cloned fragments such as the herpes TK gene as vectors or as selective markers for introducing genes into cells which are destined to be reintroduced into man.

A different approach has been taken by Cline *et al.* (this issue *op. cit.*) who have transformed normal bone marrow cells with cellular DNA and have chosen a system in which the selection of the transformed cells takes place *in vivo*, after the treated cells have been introduced into a new host. As a marker of transformation they used the dihydrofolate reductase (DHFR) gene, which if present in a large number of copies per cell confers resistance to the anti-cancer drug methotrexate, which acts by inhibiting DHFR. Selection *in vivo* is by subsequent treatment with methotrexate. This selective system allows them to use normal bone marrow cells as the recipients rather than a mutant cell line.

A few years ago R. Schimke and colleagues at the University of California, Los Angeles found that resistance to methotrexate is sometimes a result of an unusual amplification of the genes for DHFR, with consequent overproduction of the enzyme which overcomes the inhibition. As their source of amplified DHFR genes, Cline *et al.* took total DNA from a line of mouse cells in which the DHFR genes had been amplified some 30 times.

They used this to transform normal mouse bone marrow cells by a conventional calcium precipitation technique. The treated cells were injected into mice of a different but compatible strain which had been irradiated to clear the marrow of host bone marrow cells. The treated cells could be distinguished from those of the host by their distinctive chromosome pattern. Along with the treated cells were injected an equal number of 'mock' transformed host-type bone marrow cells treated with DNA from non-resistant cells.

The mice were then treated with methotrexate so that injected cells which had received large numbers of DHFR genes and were able to express them would be selected over normal host or donor cells, and, if stem cells, would multiply preferentially. If there is no selection for the added gene, both types of cell will remain in equal numbers, as the only

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difference in their lineage is the treatment of one with DNA containing large rather than small numbers of DHFR genes.

It turned out that after 30 days, the proportion of marrow cells derived from the DHFR-gene treated marrow culture was greater than 70% in most cases. The proportion continues high as long as methotrexate is administered, but when it is withdrawn the ratio returns to normal. Since the increase was also found in stem cells, this result is puzzling unless one assumes that the DHFR genes are gradually eliminated from the cell population when selection is removed. It is not yet known whether the DHFR genes have become integrated into the cellular DNA; if they are persisting in an episomal state they may be more easily lost. The mice were apparently healthy, as the levels of methotrexate used for selection were not very high. Direct analysis of the marrow cells showed a high level of the enzyme, as would be expected.

One cogent objection that could be raised to this work is that as genes for DHFR do exist in normal marrow cells, it is possible that the resistant cells could have arisen in the treated cells by the amplification of these DHFR genes rather than by the introduction of genes *in vitro*. Indirect supporting evidence that their procedure does lead to transformation and selection of transformed bone marrow cells is provided by other experiments by the same authors (submitted for publication elsewhere) in which cloned herpes TK gene sequences were inserted into mouse marrow cell cultures and these cells were transplanted into irradiated mice of a different chromosome pattern, but otherwise genetically compatible. Unlike the mouse DHFR genes, these herpes TK genes can be unambiguously recognised by their DNA restriction pattern as they are from a human virus and do not normally occur in mouse cell nuclear DNA.

The transplanted marrow did indeed contain herpes TK genes, and these appeared to be integrated in some fashion into the host cell DNA. Moreover, if the mice are treated with methotrexate (which also can select for thymidine kinase by another mechanism) the cells containing the gene are selected for in the marrow.

Bone marrow is at present the only tissue in which one might seriously consider clinical gene replacement in the near future, to correct some of the haemoglobinopathies, for example sickle-cell anaemia. Bone marrow can be relatively easily extracted, manipulated *in vitro* and replaced.

However we do not yet have the answer to the crucial question of whether it will be possible to restore correct control over the introduced genes, for it is absolutely essential that they should be expressed under the same controls as those in normal animals. Expression in inappropriate tissue or abnormal control in the cell in which they are normally expressed could be disastrous. A surplus of one globin chain

would be as harmful physiologically as a deficit, substituting an iatrogenic illness for a genetic one. Before gene therapy becomes a real possibility either ways must be found to insert the required gene into the 'correct' place on the chromosome or other methods



100 years ago

The *Photographic News* is responsible for the following:— Everybody knows how jealously the gates of the Royal Observatory are guarded, and what difficulties even scientific men have to gain admission. But Mr. Glaisher, the worthy President of the Photographic Society, and who was until lately Superintendent of the Meteorological Department, tells a story that goes far to prove that nothing is impossible to a resolute man. A vast star shower had been anticipated and its coming heralded in every newspaper. The staff at Greenwich, with the Astronomer-Royal at their head, remained the whole night through making observations and counting the bright meteors as they fell. The weary night passed, and the small hours of the morning came, only to find the jaded observers still pursuing their duty. "That makes 10,704," said our friend Mr. Glaisher. "Beg pardon; how many?" exclaimed a voice behind him. "10,704," repeated the President of the Photographic Society; and then, not recognising the voice, he turned and saw a stranger: "Who are you, and where do you come from?" At first, the only possible conjecture was that the stranger had fallen from the clouds along with the star shower; but it was not so, for, closing a little note-book, he simply replied, "I am the special correspondent of the *New York Herald*. Thank you very much. Good morning." How that special managed to get through the park gates and elude the vigilance of the keepers; how he got inside the walls of the Observatory; how he pressed into the sanctum of the Astronomer-Royal is a mystery to this day; but within a few hours of his interview with Mr. Glaisher the readers of the *New York Herald* printed a correct account of the marvellous star shower, together with many interesting details of the Observatory itself.

The French Exploring Expedition for the Trans-Saharan Railway has left Wargla for the interior of Africa.

From *Nature* 21; April 1, 524; 1880.

must be found to ensure balanced gene expression.

The final question is, why bother, particularly as antenatal diagnosis is now possible for sickle cell disease and thalassaemia? But there are still many people suffering from these diseases, and there will always be some new ones arising — this in itself justifies the approach. However, the main reason is perhaps that the haemoglobinopathies are quite unusual amongst genetic disease in that carriers can be identified easily. There are many conditions where carriers cannot be detected antenatally, and in this case treatment rather than prevention may be the only feasible strategy. □

Winds in the polar thermosphere

from Henry Rishbeth

THE observations of Smith and Sweeney, reported in this issue of *Nature*, page 437 make a new contribution to knowledge of the polar upper atmosphere — specifically the thermosphere, at heights of 200–300 km. Working in Spitzbergen, during continuous winter darkness, Smith and Sweeney used a Fabry-Perot interferometer to measure doppler shifts of the red 630 nm auroral emission. The instrument points sequentially in different directions and, with a few minutes' integration, typically measures the wind component in the line of sight to within about $\pm 20 \text{ m s}^{-1}$.

The 630 nm ($^3\text{P} - ^1\text{D}$) emission is radiated by the metastable ^1D state of atomic oxygen, which is excited by dissociative recombination of O_2^+ ions or by soft electron precipitation. With a radiative lifetime of 110 s, the ^1D atoms may be expected to take up the neutral air velocity so that the doppler shift of their radiation should correspond to the neutral air wind velocity. The technique is now well-established for measurements of nighttime thermospheric winds at mid-latitudes^{1,2}; more recently the Mawson Institute group in Australia has reported daytime measurements of thermospheric winds with the technique, obtained despite the intense background of scattered solar radiation³.

This optical technique has the drawback that the radiation originates over a range of height, within which the wind velocity may vary considerably. This problem may be real for the thermally-driven winds at lower latitudes; but in the polar thermosphere (as discussed later) the wind is mainly driven by a large-scale electric field which is almost height-independent above 200 km,

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below which there is little 630 nm emission. There might be localized winds in the vicinity of active auroral forms, but most of the Spitzbergen observations were not made in such conditions. The height variation of 630 nm emission can to some extent be studied by using a limb-scanning interferometer carried in a satellite, for example OGO-6 (ref.4); but only at the expense of horizontal resolution which is several hundred kilometres, at best. The satellite technique does however give the benefit of global coverage, within orbital limitations.

Smith and Sweeney's observations were made in the polar cap, a region usually about 30° in diameter, bounded by the 'auroral oval'⁵ in which auroral phenomena occur. Their results confirm a picture of polar thermospheric winds that has hitherto been based partly on observations by various techniques and partly on theory. Kohl and King⁶ first described the dayside-to-nightside winds across the polar cap that would result purely from heating of the dayside thermosphere by solar extreme ultraviolet (EUV), taking into account the effects of coriolis force and ion-drag (friction between ions and neutral air) on the wind direction. However, intense localized heating in the auroral oval — due partly to particle precipitation but mainly to joule heating by auroral electric currents^{7,8} — must modify the solar-driven wind pattern. Further modification results from electric fields, which at heights above 150 km produce Hall drift of the plasma ('E × B' drift), the horizontal component of this drift being imparted by ion-drag to the neutral air^{9,10}. This electrically-driven wind can be suppressed by 'back pressure' if it creates accumulations or depletions of air anywhere¹¹; however, in the polar cap the wind is nearly divergence-free and little 'back pressure' should occur¹².

The high-latitude electric fields comprise, first, intensive fields of magnetospheric origin, more or less localized in the auroral oval and peaking during substorms, and second, the

extensive dawn-to-dusk field in the polar cap that derives from the streaming of solar wind past the magnetosphere. The latter causes a dayside-to-nightside flow of ionospheric plasma across the polar cap, forming part of a great convective system with return flows at somewhat lower latitudes^{13,14}.

This 'plasma convection' has been studied in some detail by tracking chemical tracers released from rockets^{15,16}, by satellites such as Atmospheric Explorer¹⁷, and by incoherent scatter radars¹⁸; while the associated electric fields has been measured from balloons¹⁹. Smith and Sweeney's data show that the neutral air moves in a similar way to the plasma, at least for the winter conditions they studied. Around midday and midnight, the directions could probably be fitted by the Kohl and King theory⁶ based on solar EUV heating, but the convection theory¹⁵ accounts much better for the wind directions in the morning and evening sectors.

There remain many interesting problems to be resolved, not least the effect of the interplanetary magnetic field configuration on the plasma convection¹⁹. As to forthcoming activities, the previously-mentioned effects of auroral electric fields and joule heating will be intensively studied in the international campaign aimed at quantifying the upper atmosphere's energy budget, to take place in Scandinavia in winter 1980/81. Auroral motions will be increasingly studied with coherent radars such as STARE (the Scandinavian Twin Auroral Experiment). The European Incoherent Scatter (EISCAT) radar will come into operation in Northern Scandinavia and plans exist for upgrading the radar at Millstone Hill (Massachusetts) and moving the Chatanika (Alaska) radar to Greenland. Moreover, the Antarctic thermosphere differs significantly from the Arctic — largely because of the different magnetic field geometry — and future investigations may increasingly be directed southward. □

Response of plants to sulphur dioxide

from J.N.B. Bell

RECENT years have seen a reduction in the levels of SO₂ that are generally believed to inhibit plant growth. Previously it was commonly accepted that reductions in growth only occurred when relatively high concentrations ($\geq 500 \mu\text{g m}^{-3}$) caused visible injury to the foliage. It is now clear that the atmosphere in some sites where the mean SO₂ level is typical of many parts of Britain can substantially reduce the growth of grass species without any obvious symptoms on the leaves (Crittenden & Read *New Phytol.* **83** 645; 1979). Much air pollution research now concentrates on fumigation experiments designed to simulate such low SO₂ concentrations over prolonged periods and is aimed at quantifying the economic impact on vegetation of the 5.8×10^6 tonnes of SO₂ emitted annually in the United Kingdom. In practice it has proved difficult, even for the most intensively studied species, *Lolium perenne* (perennial ryegrass), to establish clear dose-response relationships and furthermore, different groups of workers have produced contradictory results with similar doses of SO₂ (see Bell *et al. New Phytol.* **83**, 627; 1979).

It is becoming increasingly apparent that the results of air pollution experiments are influenced considerably by environmental conditions within the fumigation chambers and there is circumstantial evidence that

slow growth enhances the toxic action of SO₂ (Bell *et al. op cit.*, 1979; Cowling & 'Lockyer *J. exp. Bot.* **108**, 257; 1978). Davies (this issue of Nature, page 483) provides the first unequivocal evidence that environmental conditions causing slow growth can interact with a moderate level of SO₂ to reduce yield.

Phleum pratense (timothy) was fumigated with $343 \mu\text{g m}^{-3}$ SO₂ for 5 weeks under two light regimes, representing summer and winter conditions, respectively. The SO₂ treatment caused a 50% reduction in dry matter compared with clean air controls under the winter conditions, but no effect under the summer light regime. Davies points out that many fumigation experiments are conducted under optimum growth conditions and that extrapolation to the field where there are marked environmental fluctuations, must be treated with caution.

Much of the early work with SO₂ was done in chambers with low rates of air movement, in which relatively high concentrations of SO₂ were reported to be harmless to plants. Ashenden and Mansfield (*J. exp. Bot.* **28**, 729; 1977) questioned the validity of these experiments, by demonstrating the importance of wind-speed in controlling uptake of SO₂ by vegetation. Using wind tunnels, they showed that $315 \mu\text{g m}^{-3}$ SO₂ reduced growth at a wind-speed of 25 m min⁻¹, but not at 10 m min⁻¹. They suggested that the lack of effect at the low wind-speed was caused by a failure to overcome the

- Hernandez, G. & Roble, R.G. *J. geophys. Res.* **81**, 2065 & 5173, (1976).
- Jacka, F., Bower, A.R.D. & Wilksch, P.A. *J. atmos. terr. Phys.* **41**, 397 (1979).
- Cocks, T.D. & Jacka, F. *J. atmos. terr. Phys.* **41**, 409, (1979).
- Blamont, J.E. & Luton, J.M. *J. geophys. Res.* **77**, 33534, (1972).
- Fel'dshteyn, Ya.I. & Starkov, G.V. *Planet. Space Sci.* **15**, 209, (1967).
- Kohl, H. & King, J.W. *J. atmos. terr. Phys.* **29**, 1045, (1967).
- Cole, K.D. *Nature* **194**, 42 & 75, (1962).
- Mayr, H.G. & Holland, H. *Planet. Space Sci.* **20**, 379, (1972).
- Fedder, J.A. & Banks, P.M. *J. geophys. Res.* **77**, 2328, (1972).
- Maeda, H. *J. atmos. terr. Phys.* **38**, 197, (1976).
- Dickinson, R.E., Roble, R.G. & Ridley, E.C. *J. atmos. Sci.* **28**, 1280, (1971).
- Rishbeth, H. *J. atmos. terr. Phys.* **39**, 111, (1977).
- Knudsen, W.C. *J. geophys. Res.* **79**, 1046, (1974).
- Axford, W.I. & Hines, C.O. *Canadian J. Phys.* **39**, 1433, (1961).
- Meriwether, J.W. *et al. J. geophys. Res.* **78**, 6643, (1973).
- Kelley, M.C., Jorgensen, T.S. & Mikkelsen, I.S. *J. atmos. terr. Phys.* **39**, 211, (1977).
- Heelis, R.A., Hanson, W.B. & Burch, J.J. *J. geophys. Res.* **81**, 3803, (1976).
- Doupnik, J.R. *et al. J. geophys. Res.* **77**, 4268, (1972).
- Mozer, F.S. *et al. J. geophys. Res.* **79**, 56, (1974).

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boundary-layer resistance, so reducing the flux of SO_2 to the leaf surface. At the higher wind-speed, which is more representative of field conditions, the boundary-layer resistance was overcome and the stomatal resistance became the main factor controlling SO_2 uptake.

Another problem in fumigation experiments arises from the difficulty in defining and simulating the most important characteristics of ambient air pollution. Most experiments have used a constant concentration of SO_2 , but it is possible that occasional short-term peaks, which occur in the field, may be responsible for yield reductions observed at mean ambient concentrations which are not generally considered toxic. Furthermore, the presence of other pollutants in ambient air may have positive or negative interactions with the effects of SO_2 on plant growth. Thus Brough *et al.* (*Chem. Ind.*, 21 Jan; 1978) reported a 40% reduction in grain dry weight of barley raised in chambers ventilated with ambient air containing a mean concentration of $61 \mu\text{g m}^{-3} \text{SO}_2$, compared with clean air. The proximity of a large brickworks to their experimental site probably resulted in occasional peaks of SO_2 considerably in excess of the mean, as well as the presence of atmospheric fluoride pollution.

Few attempts have been made to relate physiological effects of SO_2 to growth reductions in the field. Recently, Black and Unsworth (*J. exp. Bot.* 114, 81; 1979) have shown that only $50 \mu\text{g m}^{-3} \text{SO}_2$, a level characteristic of many rural areas, can induce reductions in net photosynthesis

and changes in stomatal resistance of *Vicia faba* (broad bean). They have further demonstrated (*J. exp. Bot.* 116, 473; 1979) that the reduction in net photosynthesis at low irradiances was independent of SO_2 concentration. On this basis it was predicted that a fully developed canopy of *V. faba*, in which the lower leaves are shaded, would show a fairly similar reduction in dry matter production, irrespective of SO_2 concentration within the range $100\text{--}500 \mu\text{g m}^{-3}$. This emphasizes the importance of a clear understanding of the influence of SO_2 on whole crop physiology when predicting effects in the field.

Work during the past decade has laid the foundations for the understanding of the long-term effects of low concentrations of SO_2 on the growth of plants and the various factors which may modify these. However, the economic impact of SO_2 injury on plants remains uncertain. There is clear evidence for the stimulation by SO_2 of plant growth on sulphur deficient soils (Cowling & Lockyer, *op. cit.*) and for the evolution of SO_2 tolerance in grass populations in polluted areas (Horsman *et al.*, *J. exp. Bot.* 30, 495; 1979). Research during the next 10 years should be aimed at quantifying the importance of SO_2 or crop growth in areas with different pollution characteristics. Field investigations must be backed up by laboratory fumigations, which should closely simulate the ambient situation with respect to climatic conditions, realistic fluctuating levels of different mixtures of pollutants, and normal practice in crop cultivation. □

reconstructed as an extensive structure like that of bats, involving also the hind limbs and tail, and the weak flight muscles would have been quite inadequate to flap this wing. Physiology and structure combined to suggest a clumsy creature that could only have soared on its great flight membranes. Furthermore, pterosaurs also seemed to have been condemned to a perpetually wandering Flying Dutchman existence, for their weak hind-limbs made it difficult to understand how they could have risen into the air once they had landed.

Research over the past 10 years has overturned all these assumptions and, though many problems remain, pterosaurs now seem rather more comprehensible. Earlier workers had seen hair-like markings on the body, but it took the discovery of a much better-preserved specimen (Sharov *Trudy pal. Inst. Akad. Nauk. SSSR* 130, 104; 1971), together with a much more open-minded approach to the physiology of extinct reptiles, before it became respectable to consider the possibility that pterosaurs were insulated and, perhaps, homiothermal.

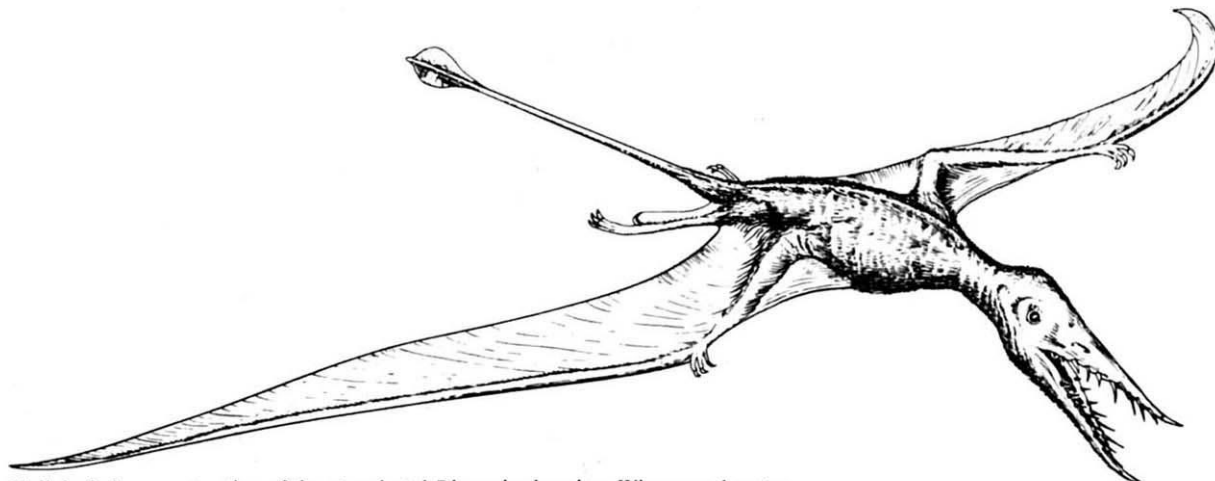
Studying the skeletons and wing-impressions of the smaller pterosaurs, known as pterodactyls, Wellnhofer (*Abh. bay. Akad. Wiss.* 141, 1; 1979) showed that there was no evidence that the wing was attached to the hind foot. He therefore restored the wing with a much narrower, bird-like outline (see illustration), and Padian (*Discovery* 14, 20; 1979) has recently shown that, if the estimated body-weight of a pterosaur is compared with the body-weight : wing area relationships of living animals, the results also suggest a smaller, more slender flight membrane. Such a wing, freed from attachment to the hind limb, is obviously more manoeuvrable in flight and less cumbersome when the wing is folded and the animal is moving on the ground. Wellnhofer also dismisses the belief that there was a flight-membrane between the hind legs and the tail. The resulting slim-line pterosaur will necessitate the reworking of previous calculations on their wing-loading and aerodynamic capabilities (for example Bramwell & Whitfield *Phil.*

Trimming the pterosaur's wings

from Barry Cox

THE old-fashioned view of the extinct flying reptiles known as the pterosaurs seemed reasonably consistent. Being reptiles, they were 'obviously' cold-

blooded. The lack of any high keel running along the sternum suggested weak wing muscles. The flight membrane, borne on the arm and elongated fourth finger, was



Wellnhofer's reconstruction of the pterodactyl *Rhamphorhynchus*. Wing span 1 metre.

THE recent papers by Gleadow (*Nature*, **284**, 225; 1980) and McDougall *et al.* (*Nature*, **284**, 230; 1980) may have ended a decade of controversy over the age of the KBS tuff in the East Turkana region of Kenya. The KBS tuff is critical to the dating of Early Man and his tools in this important anthropological site. Efforts to date this tuff provide a fascinating and instructive case history of geochronology which illustrates the process of trial and error by which science progresses. Some of the highlights of this case history are sketched below.

The KBS tuff lies near the middle of the Koobi Fora Formation, which yielded its first hominid fossils to Richard Leakey's expedition in 1968. By 1973, 110 hominid fossils had been collected. Primitive artefacts were discovered in 1969, and two occupation sites were excavated in the KBS tuff by Isaac *et al.* (*Science* **173**, 1129; 1971). Feldspar from the tuff was dated by Fitch and Miller (*Nature*, **226**, 226; 1970) both by the conventional (total degassing) K-Ar method and by the newly developed $^{40}\text{Ar}/^{39}\text{Ar}$ step-heating method. The conventional method produced a wide scatter of ages, clearly in part because of contamination by older feldspar. An age of 2.61 ± 0.26 Myr was assigned to the tuff on the basis of the $^{40}\text{Ar}/^{39}\text{Ar}$ age spectra. Dates younger than 2.6 Myr from the conventional method were believed to reflect argon loss caused by regional hydrothermal alteration.

Doubt about the date of 2.6 Myr was raised by some palaeontologists, who found an age of 2 Myr more in accord

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The KBS tuff controversy may be ended

from R. L. Hay

with dated faunas at Olduvai Gorge and the Shungura Formation in the Omo basin (Cooke & Maglio in *Calibration of Hominoid Evolution* (eds Bishop, W. E. & Miller, J. A.) 303, Scottish Academic Press; 1972). The palaeomagnetic stratigraphy of the Koobi Fora Formation was now studied by Brook and Isaac (*Nature* **234**, 344; 1974) with the object of dating the strata by matching their polarity with the newly established polarity time scale. They found two possible fits with the polarity scale, one of which was compatible with the KBS tuff date of 2.6 Myr. Curtis *et al.* (*Nature*, **258**, 395; 1975) added to the controversy by reporting K-Ar dates of 1.60 Myr for the KBS tuff at one locality and 1.82 Myr from the tuff at another locality. Their results were particularly significant because dates on feldspar and glass were concordant, thus providing strong evidence against loss of radiogenic argon. If their results were accurate, the KBS tuff must be in fact two tuffs, separated in time by 200,000 years.

Next, zircons from the KBS tuff were dated by the fission-track method. The zircons contained low fission-track densities, but an age of 2.44 ± 0.08 Myr was obtained by Hurford *et al.* (*Nature* **263**, 738; 1976). Fitch *et al.* (*Nature* **263**, 740; 1976) recalculated their step-heating results and now suggested 2.42 Myr as the best K-Ar age.

In 1977 Harris and White (*Science* **198**,

13; 1977) concluded from their studies of suid evolution that deposits below the KBS tuff were equivalent to fossiliferous beds in the Omo basin and Olduvai Gorge that had been dated at about 1.8 Myr. Hillhouse *et al.* (*Nature* **265**, 411; 1977), in another palaeomagnetic study of the Koobi Fora Formation, found that although two correlations were possible with the polarity time scale, placing the KBS tuff in the Olduvai normal event (~ 1.7 – 1.85 Myr) created fewer difficulties in the geological history than did an age of 2.4 Myr for the tuff.

Cerling *et al.* (*Nature*, **279**, 118; 1979) now correlated the KBS tuff with tuff H₂ (~ 1.8 Myr) of the Shungura Formation on the basis of chemical composition of glass and feldspar. This correlation received strong support from additional dating of the KBS tuff by Drake *et al.* (*Nature* **283**, 368; 1980). They gave an age of 1.8 ± 0.1 Myr for the tuff, and explained that the date of 1.60 Myr reported earlier for the tuff at one locality was incorrect because of an error in K analyses.

The latest and perhaps the final statements on the age of the KBS tuff are provided by McDougall *et al.* Their meticulous K-Ar dating of feldspar gave an age of 1.89 ± 0.01 Myr. Fission-track dating by Gleadow yielded an age of 1.87 ± 0.04 Myr, and made a notable contribution to the methodology of fission-track dating of zircons which contain low track densities. In closing, it should be emphasized that the KBS tuff has been a testing ground for various geochronologic methods, both new and old, and the science of geochronology has learned much from the "KBS tuff controversy."

Trans. R. Soc. **B267**, 503; 1974). These studies have involved the great albatross-like soaring pteranodontids of the Cretaceous, such as a 7 m wing-span *Pteranodon*, but even this is dwarfed by *Quetzacoatlus* from Texas, the humerus of which is 70% longer than that of *Pteranodon*, and which may have had a wing span of 15 m!

Modern workers seem agreed that the smaller pterosaurs, at least, were actively-flapping fliers, and it now appears that there was, after all, a strong ventral keel projecting forwards from the sternum, which was itself also strongly braced from the scapula by powerful coracoid bones, very much as in birds.

The freeing of the hind-limb of pterosaurs from the flight membrane also helps in the interpretation of their method of launching themselves into the air. Wellnhofer (*Palaeontographica* **A149**, 1; 1975) believes that the hind-limbs could at least have raised the body into the air far enough that, facing into the wind, the outstretched wings could lift the body from the ground, and that the hind-limb could

also have helped to thrust it skywards.

But further problems arise from the diet of pterosaurs. Both their common presence in marine deposits, and their preserved stomach contents, show that most of them fed on fish. Wild has most recently discussed the problems of the method they used to catch their prey (*Boll. Soc. pal. Italiana* **17**, 176; 1978). Though some living birds catch fish from near the water surface by flying close above it, Wild points out that pterosaurs must have been far less competent aerodynamically. This, together with their proportionately much longer wings, would make it inevitable that a wing-tip would sooner or later hit the water, bringing the whole creature down into the sea. Wild also argues that the braking effect of dipping the beak into the water to catch fish would cause such a drop in speed as to bring the pterosaur down. He instead believes that they fished by folding their wings and diving into the water. (This would inevitably have placed considerable strain on their skull and skeleton, and it

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would be instructive to investigate whether they show any convergences with the adaptations of living diving birds such as gannets.)

There is evidence that the hind feet of pterosaurs were webbed and they must have used these for swimming, whether their immersion was voluntary or accidental. Wild thinks it possible that they might have been able to rise from the water surface by spreading their wings, or that they might instead have swum to nearby land so as to rise from a solid surface. (Could it be that the hairy covering had a water-repellent function?)

The pterosaurs which Wild describes come from the Late Triassic and include members of two different families. The origin of the group as a whole must therefore lie in the earlier Triassic at least. Wild points out that the whole organization of the early pterosaurs suggests derivation from a small running and climbing insectivorous reptile. He believes that this strongly suggests that they evolved from the little eosuchians of the Late Permian and Early Triassic, rather

than from the larger terrestrial carnivorous pseudosuchians, most of which were in any case too late in time to be potential ancestors.

Though some problems have then, at

least been tackled, one still cannot help feeling that just as a camel is said to be a horse designed by a committee, the pterosaur is the result of that committee turning its attention to birds. □

Fish vision discussed

from a Correspondent

THE thirty thousand or so living species of fishes inhabit a wider variety of visual environments than any other vertebrate group. Different water bodies vary widely in their colour and turbidity, presenting very different visual tasks to their inhabitants; many species migrate during their life span exposing themselves to these various environments; and there are also often marked seasonal changes in the water's quality. The fishes, therefore, form an ideal group for comparative studies of vision. If we add to this the fact that they are readily available and excellent subjects for physiological experiments, it is not surprising that a great deal of work has been done on various aspects of their vision. Some was reported at a recent meeting of the British Photobiology Society held in London*.

Anatomical papers on the retina (H.-J. Wagner, Universität Ulm) and on cone photopigments (J.N. Lythgoe, University of Bristol) made full use of the comparative approach, and showed that striking differences, clearly associated with the environment, may be found. Explaining their significance is, however, often more difficult. Why, for example, should fish living near the surface be less sensitive to long wavelength light than those that live deeper? They clearly are, though no one knows the reason.

K.H. Ruddock (Imperial College, London) presented results from experiments involving strong bleaching of the retina with a laser, a technique that can reveal receptor interactions that are normally concealed. R. Weiler (Universität München) then described his results on horizontal cells, which posed the unanswered conundrum of how a graded potential can be transmitted without degradation along 600 µm of axon when theoretical considerations suggest 150 µm should be the maximum possible distance. M.B.A. Djamgoz (Imperial College, London) and S.H. Reynolds summarized their work on possible retinal transmitter substances. A wide variety of possible transmitters exist: a list of some 30 candidates was given, of which the most likely ones are GABA and aspartate. Although, however, the evidence is very suggestive, even in these cases it is not conclusive and much work, which should

prove very profitable, clearly remains to be done.

These retinal studies used fish mainly because of their experimental convenience. Reports on the anatomy and physiology of the optic tectum followed in which fish were also used largely for this reason. In the complex analyses that the tectum carries out, however, the problems of the aquatic environment also began to reappear. For example, tectal units responding in a most striking way to very short wavelength light were described for the perch, which is surprising in a fish that does not possess blue cones, has a yellow cornea, and lives in

coloured water in which short wavelength light is almost totally absent. Other units that respond preferentially to repeated stripes were described for this species: could these be related in any way to the fact that the perch itself is striped?

Two papers reported laboratory behavioural studies on spectral sensitivity in fish. W.R.A. Muntz (University of Stirling) reported data that compared vision in an upward direction with vision downward: these showed marked differences in both absolute sensitivity and the form of the spectral sensitivity curve, which may plausibly be related to the difference in the amount of light reaching the fish from the two directions. C. Neumeier (Institut für Zoologie, Mainz) presented spectral sensitivity data for goldfish. One striking and confusing fact about behavioural studies is that whenever the experimental situation is changed the results also usually change, and these results were no exception to this rule. Presumably different neurones, with different sensory inputs, contribute preferentially to different behaviour patterns. □

Polarity of spindle microtubules

from Jeremy Hyams

UNLIKE microfilaments in which directionality can be visualised directly by specific interaction with heavy meromyosin, the polarity of microtubules has, until now, been approachable only by more devious methods. The most successful has exploited the ability of tubulin subunits with attenuated capacity to self-assemble, to polymerize *in vitro* onto some preformed seed or organizing centre. Various cellular structures will apparently serve although most frequently used are flagellar axonemes from the green alga, *Chlamydomonas*, whose two ends are morphologically distinguishable. When axonemes are incubated with low concentrations of brain tubulin (<2 mg ml⁻¹), microtubules grow by the addition of subunits onto the distal end only. At higher tubulin concentrations, growth is onto both ends of the axoneme although the rate of subunit addition at the distal end is at least three times that at the proximal (Allen & Borisy *J. molec. Biol.* **90**, 381; 1974; Binder *et al. Proc. natn. Acad. Sci. U.S.A.* **72**, 1122; 1975). This directional polymerization is taken to reflect an intrinsic molecular polarity within the microtubule structure, that is, the asymmetric tubulin subunits are all oriented in the same way along the microtubule axis, an arrangement also inferred from detailed analyses of microtubule structure by electron microscopy and optical diffraction (Amos & Klug *J. Cell Sci.* **14**, 523; 1974).

Microtubule polarity has been a central concern in studies of mitosis ever since a suggestion made over a decade ago by McIntosh, Hepler and van Wie (*Nature* **224**, 659; 1969), that the polar functions of the mitotic spindle in mammalian cells might reflect an antiparallel relationship of microtubules originating from the polar centrosomes and the chromosomal kinetochores. The subsequent demonstration by many workers that both of these components would nucleate the assembly of neurotubulin in cell-free systems (McGill & Brinkley *J. Cell Biol.* **67**, 189; 1975; Snyder & McIntosh *J. Cell Biol.* **67**, 744; 1975; Telzer *et al. Proc. natn. Acad. Sci. U.S.A.* **72**, 4023; 1975) appeared, at least superficially, to be consistent with the McIntosh *et al.* model.

A more recent series of papers from Margolis and Wilson, however, has struck a discordant note. On the basis of an analysis of the exchange of [³H]GTP with microtubules assembled to steady state, these workers have proposed a novel mechanism of microtubule assembly whereby subunits are added to one end of the microtubule and removed at the other in a unidirectional (opposite end) assembly and disassembly process (*Cell* **13**, 1; 1978). The result is a continuous flow, or 'treadmilling' of subunits along the microtubule at a rate measured to be 0.69 µm h⁻¹. Extrapolation of these findings to

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*The Visual System of Fish, organized by M.B.A. Djamgoz (Imperial College, London) was held at Imperial College on 27 February, 1980.

the microtubules of the mitotic spindle has yielded a picture of spindle organization which differs radically from that outlined above (Margolis *et al.* *Nature* **272**, 450; 1978). Briefly, Margolis *et al.* envisage that microtubules assembled at the poles grow by the addition of subunits at the end distal to the organizing centre, while those assembled at the chromosomes are extended by the insertion of subunits proximal to the kinetochore. The result is a spindle in which the microtubules reveal a parallel orientation either side of the equator. Such an arrangement is not without appeal. The continuous insertion of subunits to both chromosomal and polar microtubules in the equatorial region and their subsequent removal at the poles might be expected to produce a constant poleward migration of material, a phenomenon often seen in the spindles of living cells. Although the measured rates of subunit treadmill in *vitro* are generally lower than motions associated with living spindles, more recent work from the same laboratory has shown that the *in vitro* rate can be increased some 20-fold by the addition of 1 mM ATP to their assembly soup, giving a rate more consistent with that *in vivo* (Cell **18**, 673; 1979).

Unfortunately for such an elegant concept, the model was destined for a relatively short half-life, a situation by no means without precedent in the mitosis literature. Its demise was signalled by the appearance of two papers from the University of Wisconsin (Bergen & Borisy *J. Cell Biol.* **84**, 141; 1980; Bergen *et al.* *J. Cell Biol.* **84**, 151; 1980). Following an earlier suggestion from the same laboratory (Borisy *J. molec. Biol.* **124**, 565; 1978), these workers have undertaken a detailed analysis of the kinetics of polymerization and depolymerization of microtubules initiated *in vitro* by the centrosomes and kinetochores of Chinese hamster ovary cells. As a unique added refinement, Bergen and his colleagues have included in their analysis, *Chlamydomonas* flagellar axonemes as an unambiguous internal marker of directional microtubule growth. Thus it was possible to determine whether microtubules assembled onto the spindle organizers did so at a rate equivalent to that seen at either the proximal or distal ends of the flagellar standards alone, or alternatively at a rate which was a summation of the two reactions. The results were unequivocal. The rate of assembly into the mitotic organizers was identical to that at the distal end of the axoneme only, irrespective of whether conditions also supported the proximal extension of the internal standard. Equally important, a corresponding survey of the rate of depolymerization showed that this too was equivalent solely to the reaction taking place at the distal end of the added axonemes. Both findings conflict with the view of the spindle proposed by Margolis *et al.* in several important respects. Both

polar and chromosomal microtubules grow by addition of subunits distal to their site of initiation. Their arrangement in each half of the spindle must consequently be antiparallel. Finally, addition and removal of subunits takes place at the same, and not opposite, ends of the microtubule.

Intriguing as these assembly studies might be, there are encouraging signs that their days as the sole experimental handle on microtubule directionality are numbered. An exciting paper by Haimo, Telzer and Rosenbaum (*Proc. natn. Acad. Sci. U.S.A.* **76**, 5759; 1979) suggests that the long-awaited morphological marker of microtubule polarity might at last be at hand. The candidate is dynein, the ATPase protein responsible for the movement of cilia and flagella and seen as paired projections, or arms, connecting the 9+2 microtubules. Haimo and her colleagues have shown that axonemal dynein from *Chlamydomonas* added to microtubules during or subsequent to assembly binds periodically along the length of the microtubule, forming a complex reminiscent of the 'decoration' of

microfilaments by heavy meromyosin. The centre-to-centre spacing of the dynein projections is 24 nm, the same as its periodicity in the flagellum from which it derives. More important, when these reconstituted dynein arms were examined in the electron microscope they were characteristically tilted when viewed in longitudinal section and hooked when seen in transverse profile. Thus the arms can be used to define microtubule polarity in either orientation.

Although it is a long step from these *in vitro* studies to the *in situ* decoration of microtubules like those of the mitotic spindle, such an application might prove to be particularly appropriate. The anaphase movement of chromosomes in lysed mammalian cells has been shown to depend on the presence of ATP (Cande *et al.* *Proc. natn. Acad. Sci. U.S.A.* **71**, 573; 1978). Whether dynein is eventually shown to be the force-generating component of the spindle remains to be seen. What is clear is that this protein is going to have a central role in an exciting new era of microtubule research. □

The metal-semiconductor contact

from D.C. Northrop

METAL-semiconductor contacts (Schottky barriers) are commonplace in modern semiconductor electronics. Not only do they perform the active function of rectification in devices as widely different as microwave detectors and high current rectifiers, but they serve as ohmic contacts to virtually every semiconductor device that is made. Because of their evident importance such contacts continue to provoke widespread research interest on two quite separate fronts. One of these concerns the yield and reliability of semiconductor devices and microcircuits, where the metal-semiconductor contact and its associated metal wire bond are the most fallible part of the whole system. The other area of research, and the one that concerns us here, is the search for understanding of the physics of the contact; the relationship between its electrical properties and its structure.

Historically, the earliest attempt to provide a physical model for potential barrier formation was that commonly attributed to Schottky (Schottky *Naturwissenschaften* **26**, 843; 1938), but more correctly credited to Mott (Mott *Proc. Cambridge Phil. Soc.* **34**, 568; 1938) in the form in which it is usually stated. This model proposes that the electrostatic potential barrier opposing electron flow from the semiconductor to the metal is equal to the difference between the work

functions of the metal and the semiconductor making up the contact. This has an elegant simplicity about it and held sway for many years, partly because work functions are notoriously hard to measure experimentally, making the theory difficult to check. The first workers to provide adequate experimental results for metal contacts to clean silicon were Allen and Gobeli (*Phys. Rev.* **127**, 150; 1962) who found barrier heights that were essentially independent of the work function of the metal, apparently confounding the theory. Their results led to the acceptance of an idea proposed earlier by Bardeen (*Phys. Rev.* **71**, 717; 1947). He added to the Mott-Schottky model the concept of surface states; localised energy levels at the surface, whose electron occupancy had a controlling effect on the electric field in the semiconductor. This allowed a qualitative explanation of Allen and Gobeli's results, and also accounted for the great variability of other less carefully obtained experimental data.

Then followed the longest and most difficult phase of the work. Experimental techniques improved in every way; better vacuum systems, a greater number of very pure metals and semiconductors and more sensitive measuring techniques have all contributed to the search for these elusive surface states. Theoretical physicists have been at work too, and have played an important part in guiding experimental work. Particularly influential in this area have been Heine (*Phys. Rev.* **138A**, 169; 1965) and Inkson (*J. Phys. C* **6**, 1359;

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1973), who pointed out that when metal and semiconductor are placed in intimate contact their surfaces will both be changed, particularly by the ability of electrons to penetrate from the metal into the surface of the semiconductor by tunnelling into the forbidden energy gap. This effect changes the charge distribution and therefore the electric field at the interface in a way that could explain qualitatively quite a large body of results. Gradually it has become clear, however, that quantitatively the effects predicted are not large enough to explain the number nor the spatial distribution of surface states. The results of Thanailakis (*J. Phys. C*, **8**, 655; 1975), are amongst the most important in establishing this point.

We come now to the latest suggestion, backed by extensive and impressive experimental data from Spicer and his group at Stanford University. The latest paper (Spicer *et al. Phys. Rev. Lett.* **44**, 420; 1980) spells out the mechanism they propose for barrier formation in a number of compound semiconductors. These are materials which exhibit no intrinsic surface states within the forbidden energy gap. That is, on the cleaved surfaces of these materials the Fermi Energy is found, by

photoelectric emission measurements, to be the same as that in the bulk, showing that there is no electric field in the semiconductor surface. The evaporation of metal, or the adsorption of oxygen, on these surfaces is found to change this situation and to give rise to surface states and, consequently, to electric fields which move the band edges relative to the Fermi Energy. The most important feature of their results is that the energy levels so produced are specific to the semiconductor and not to the material deposited. Spicer's explanation of this depends on the large heat of adsorption of materials on semiconductor surfaces, about 3 eV per atom according to some measurements. This is enough to produce lattice defects such as vacancies or more complex entities in and near the semiconductor surface, and it is these defects, Spicer claims, which control the potential barriers. It is perhaps early to say that the full picture has now been revealed, but it is clear that an important new step has been taken. It will lead to a better appreciation of what is possible in the production of metal-semiconductor contacts with tailored properties as well as improving our understanding of interface physics. □

virus equivalent of SV5-induced HN antibodies), and would provide additional antigenic stimulation resulting in an anamnestic response to the H antigens to which the vaccine provided the primary response. In these conditions, immune complexes composed of the antigen-producing syncytium and H antibodies could form, activate the complement system and result in inflammation and tissue necrosis. Alternatively, antibody-mediated cytotoxicity might cause the observed inflammation without complement activation. Either process could account for the immunopathological reactions that have been observed in some people given RS or measles vaccines.

In addition to providing an insight into the possible mechanisms involved in the complications associated with paramyxovirus vaccination, the work of Merz *et al.*, together with earlier work by Waldman and Ganguly (*J. infect. Dis.* **130**, 419; 1979) and McIntosh *et al.* (*J. infect. Dis.* **138**, 24; 1978), among others, suggests an approach to paramyxovirus vaccination that may be both safe and effective. Merz *et al.* show that pure F glycoprotein would be the ideal immunogen for a paramyxovirus vaccine and the earlier studies suggest that the most effective way of administering this immunogen would be directly into the lung as an aerosol. The advantage of such local immunization is that it would stimulate secretory IgA, which does not activate the complement system or antibody-mediated cytotoxicity, and would thus avoid the immunopathological consequences resulting from stimulation of IgG antibodies. The disadvantages of the suggested approach are the difficulty of developing high IgA titres and the short duration of immunity. However these problems are not insurmountable. Morein *et al.* (*Nature* **276**, 716; 1978), for example, were able to prevent a lethal infection of mice with Semliki Forest virus by vaccinating the mice with a micellar aggregate of the virus spike protein. Interestingly, they found that the monomer form of the protein solubilized with detergent was much less effective than the aggregated protein. Those results suggest that protective and lasting immunity to paramyxoviruses might be stimulated by administering the F glycoprotein as an aggregate rather than in the solubilized form. Alternatively, the F glycoprotein could be administered together with an immunoadjuvant, such as the purified active component of Freund's adjuvant, N-acetyl-muramyl dipeptide (Azuma *et al. Infect. Immun.* **14**, 18; 1976; Chedid *et al. Proc. natn. Acad. Sci. U.S.A.* **73**, 2472; 1976), which does not produce the abscesses associated with the complete Freund's adjuvant. Using either approach, it might be possible to introduce pure F glycoprotein into the lung as an aerosol and stimulate immunity without adverse side effects. If so, it would be an important step towards controlling respiratory infections.

Vaccination against paramyxoviruses

from A.J. McClelland

PARAMYXOVIRUSES are now known to be a major cause of respiratory illnesses but, despite the importance of these diseases, little progress has been made in their control. Live virus vaccines are available for only two paramyxovirus diseases (measles and mumps) and failures of these vaccines resulting in severe complications, such as modified and atypical measles, have been reported (Chatterji & Marikad *J. Am. Med. Assoc.* **238**, 2635; 1977). Inactivated virus vaccines against the measles virus and other paramyxoviruses, such as the respiratory syncytial (RS) virus and the parainfluenza viruses, have had limited success and have sometimes induced severe forms of the illnesses they are supposed to prevent (Kim *et al. Am. J. Epidemiol.* **39**, 422; 1969; Welliver *et al. Arch. Int. Med.* **137**, 39; 1977). The unusual complications that sometimes occur after paramyxovirus vaccination have been attributed to an aberrant immune response, but it is not known how or why such a response occurs.

New light has recently been shed on the problems associated with paramyxovirus vaccination by Merz *et al.* (*J. exp. Med.* **151**, 275; 1980) who have investigated the infectious spread of one paramyxovirus, SV5, using monospecific antibodies to the two SV5 surface glycoproteins. These

authors report that SV5 can be disseminated in two ways: (1) release of infectious virus from infected cells and (2) cell fusion, in which no infectious virus need be released. They found that antibodies to the viral haemagglutinating and neuraminidase (HN) glycoprotein prevent the spread of infection by the release of virus particles, but do not prevent the spread of infection by cell fusion, whereas antibodies to the viral fusion (F) glycoprotein prevent the spread of infection by both cell fusion and release of infectious virus. The results obtained by Merz *et al.* emphasize the importance of the F glycoprotein in the dissemination of SV5.

Their findings, together with the earlier observation that vaccination elicits a weak F antibody response, or fails to stimulate F antibody production altogether (Norrby & Gollmar *Infect. Immun.* **11**, 231; 1975), suggest a possible explanation for the severe complications that sometimes result from paramyxovirus vaccination. In a person vaccinated against measles, for example, exposure to the measles virus could result in a disseminated infection because of the lack of F antibodies and the ability of the virus to spread from cell to cell by cell fusion. As the infection spreads, syncytium formation would take place and the antigen load would increase. Released virus particles would be neutralized by the vaccine-induced H antibodies (the measles

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REVIEW ARTICLE

Fluorescently labelled molecules as probes of the structure and function of living cells

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A new approach to cell biology has been created by combining the techniques of micromanipulation of cells, fluorescence spectroscopy and low level light detection. These methods are now being used to study the spatial and temporal distribution, interaction and activity of specific molecules in living cells.

CELL functions such as locomotion, cell recognition, endocytosis, exocytosis, and cell division involve highly dynamic and transitory molecular interactions. Early cell physiologists studied these functions by probing living cells and developed sophisticated techniques for micromanipulation and microinjection¹⁻⁴. A new approach bridging the gaps between cell physiology, biochemistry and ultrastructure is required to define the molecular mechanisms of complex cell functions. Such an approach would require a combination and coordination of (1) techniques for manipulating living cells, (2) techniques which provide signals from specific molecules with high sensitivity, and (3) techniques which detect weak signals from living cells. Recently, the methods of micromanipulation of cells¹⁻⁵, fluorescence spectroscopy⁶⁻⁸, and low-level light detection⁹⁻¹³ have been combined to provide such a new approach to cell biology. This new concept involves labelling purified molecules covalently with fluorescent probes, and incorporating the fluorescent conjugates into or onto living cells. Cells with associated fluorescent conjugates are then either viewed with an image intensifier coupled to a microscope or are investigated with a microspectrofluorometer. Therefore, the high sensitivity ($\sim 10^5$ – 10^6 molecules can be detected¹⁴), and versatility⁶⁻⁸ of fluorescence techniques can be fully utilised in living cells to yield information at the molecular level.

This review is limited to applications of this approach in which purified molecules are labelled, administered and then studied at the light microscopical level. The technical aspects will be discussed in detail since this new approach requires careful application and interpretation. In addition, three categories of fluorescent conjugates will be discussed: (1) nonperturbing indicators of physiological processes, (2) biologically active agents and (3) functional cellular components (molecular cytochemistry)¹⁵. Some of the studies referenced could be included in more than one of these categories.

Experimental methods

The application of fluorescent conjugates to studies in single living cells demands the use of highly fluorescent fluorophores which absorb in the visible spectrum, so that adequate signals are obtained while minimising radiation damage and interference from autofluorescence¹⁶. Furthermore, the conjugates must be associated by stable covalent bonds and be devoid of non-covalently bound fluorophores. Recent advances in the preparation of fluorescent reagents have provided several compounds suitable for applications *in vivo*. Fluorochromes such as fluorescein¹⁵⁻¹⁷, eosin¹⁸, 7-chloro-4-nitrobenzo-2-oxadiazole (NBD)¹⁹, and a series of long-wavelength rhodamine dyes^{15,20-21} have been used successfully with living cells. In addition, various reactive derivatives of these fluorophores such as iodoacetamide¹⁵, isothiocyanate²⁰ and sulphonyl chloride¹³ can be obtained commercially. Many of the long-wavelength

fluorophores used primarily by neurophysiologists should be useful when reactive derivatives become available²².

Classical microinjection techniques are still the most direct methods for incorporating exogenous components into living cells^{1,23}. Several different microinjection systems, using both hydraulic pressure^{4,5,17} and compressed air^{13,24,25}, have been described in detail. Cells ranging in size from giant protozoans ($\sim 600 \mu\text{m}$) to human fibroblasts ($\sim 15 \mu\text{m}$) have been successfully microinjected. These techniques are time consuming and the numbers of cells which can be studied are limited. However, they have the advantages that only a very small volume of material is required for each experiment; components as large as

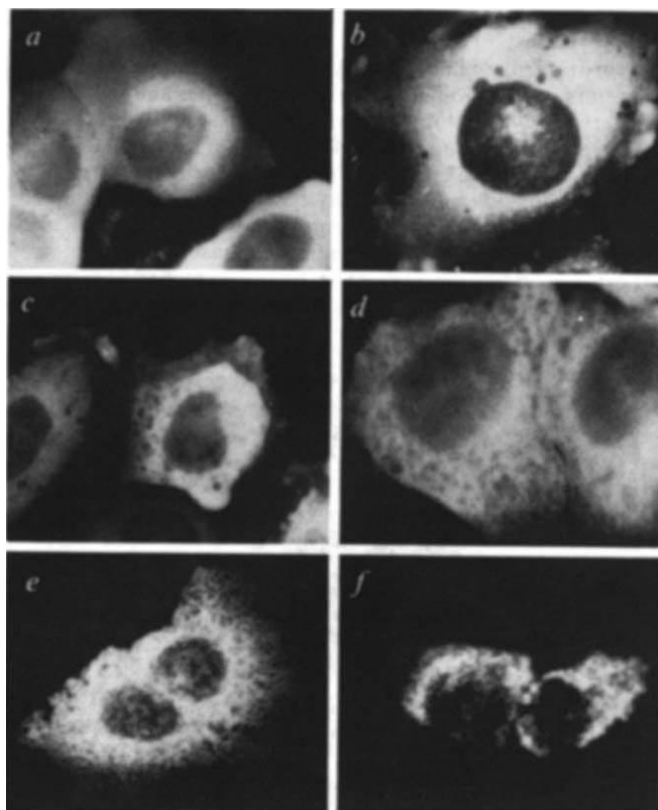


Fig. 1 Autophagy studied by injecting highly concentrated tetramethylrhodamine isothiocyanate labelled bovine serum albumin into HeLa cells. These fluorescence micrographs were taken of injected cells *a*, immediately; *b*, 30 min; *c*, 1 h; *d*, 90 min; *e*, 6 h thereafter. The labelled proteins were taken up into vesicles which ultimately fused with lysosomes (*f*). (From Stacey and Allfrey⁴⁵.)

organelles can be microinjected⁴, mild solution conditions can be used to deliver the fluorescent conjugates^{13,15,17,20}, and injection volumes can be controlled.

During the past few years, new delivery methods using fusion techniques have been developed. The molecules are trapped inside carriers such as red blood cell ghosts²⁶ and liposomes^{27,28} which are subsequently fused with target cells. While these methods allow the 'ultramicroinjection' of large populations of very small cells there are also several limitations: (1) relatively large volumes of fluorescent conjugates are required during the loading process, (2) the conjugates could become exposed to detergents, elevated temperatures, or organic solvents, (3) the proper entrapment and fusion steps must be carefully verified, and (4) the biological effects of the carriers must be controlled. Recently, fibroblasts have been successfully loaded with fluorescently labelled antibodies by red blood cell fusion techniques²⁹. Continued improvements in fusion technology should make these approaches more generally useful in the future.

Cell perfusion techniques have been used to deliver small ions and molecules into plant cells³⁰. A membrane permeation technique has now been reported which permits the incorporation of small molecules into living animal tissue culture cells³¹. The cells are permeabilised by short treatments with lysolecithin which make the cells leaky to exogenous molecules in the medium for a short period of time. This technique has advantages similar to those of fusion methods, yet does not require the use of carriers. Unfortunately, the application is limited to only very small molecules (molecular weight below 10,000) and to cells grown in monolayer culture. In addition, the cells remain viable for only a short period.

Recent developments in image intensification techniques have provided a more sensitive way of recording fluorescent images than classical photographic methods¹⁰. The use of standard photographic procedures requires long exposure times and intense illumination. Therefore, fluorescence photobleaching could be extensive, cell damage is possible, and dynamic processes are difficult or impossible to record. In contrast, TV image intensifiers coupled to fluorescence microscopes and video tape recorders allow the continuous recording of weakly fluorescent images in real time or time lapse without significant losses in resolution¹⁰. Some TV cameras also provide digital output for quantitative image analyses. When these intensifiers are coupled to optical prisms and multichannel analysers, they can also provide rapid microspectrofluorometric measurements^{10,32}.

Microspectrofluorometers have been developed for quantitative measurements of many fluorescence parameters with very low light intensities^{33,34}. In particular, photomultipliers

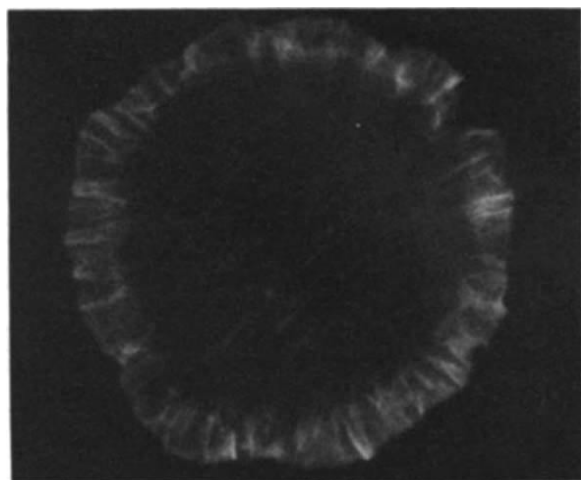


Fig. 2 Fluorescence photograph of a live chick embryo fibroblast that is lysolecithin permeabilised and labelled with NBD-phalloidin¹⁹. Most of the labelled actin in this spreading cell is located peripherally in the area of membrane ruffling. Some longitudinal stress fibres are also evident. (Micrograph courtesy of L. Barak.)

PURE ACTIN

Label with 5-iodoacetamidofluorescein

SELECT FUNCTIONAL AND OPTIMALLY LABELLED ACTIN

- (A) Polymerisation-depolymerisation
- (B) DEAE chromatography

CHARACTERISE LABELLED ACTIN

- (A) Dye/protein
- (B) Site of labelling
- (C) Polymerisability
- (D) Activation of myosin Mg^{2+} ATPase

DETERMINE FUNCTIONAL ACTIVITY IN VITRO

- (A) Single cell models
- (B) Bulk extracts

INCORPORATE LABELLED ACTIN INTO LIVING CELLS BY MICROINJECTION

Limit: 10% of endogenous actin concentration

DETERMINE FUNCTIONAL ACTIVITY IN VIVO

CORRELATE DISTRIBUTION OF ACTIN FLUORESCENCE WITH OVALBUMIN CONTROL

Fig. 3 Flow diagram of the steps involved in molecular cytochemistry. Actin is described as an example.

interfaced with single photon counting devices have provided instruments of high sensitivity³⁵. These systems in combination with computer-controlled spectral scanning and data processing form the core of several sophisticated microspectrofluorometers which are capable of performing corrected spectral measurements on single living cells³⁵⁻³⁸. The use of lasers for excitation has also increased the versatility of microspectrofluorometers, because the state of polarisation, spot size, duration and intensity of the exciting light pulse can be controlled over large ranges.

Recently, fluorescence photobleaching recovery techniques have been introduced to measure the mobility of surface associated fluorophores³⁹⁻⁴². These methods utilise a pulsed high-intensity laser microbeam to bleach a small area or volume of fluorescence, and the recovery of fluorescence is monitored with attenuated laser excitation. These measurements can yield diffusion constants of the mobile fraction, the percentage of fluorescent conjugates that are mobile, and information on the bulk directional flow of the conjugates. However, caution must be exercised in controlling the possible biological effects of photobleaching⁴³, and consideration must be given to the validity of the assumption that cell surfaces are flat and smooth. In addition, care must be taken to ensure that the membrane or surface markers are not internalised before making measurements. These potential problems have recently been addressed critically by Elson and Yguerabide⁴⁰.

Physical and chemical considerations

There are several possible physical and chemical problems which could give rise to experimental artefacts, and investigations using this new approach must be judged in part by the manner in which potential problems are considered. Factors which could affect the fluorescence intensity measured for the microscope include: (1) local accessible volume for the conjugate in the cell, (2) local environment of the conjugate, (3) optical properties of the microscope, and (4) local concentration of the conjugate.

Consideration of the local accessible volume is important for both cytoplasmic and membrane-associated components. For cytoplasmic components, the local accessible volume is controlled not only by the actual thickness of the cell, but also by the distribution of organelles which exclude the fluorescent conjugates. For membrane-associated components, the accessible volume is affected dramatically by the presence of membrane folds and microvillar structures. This problem can be

identified and controlled by using a second fluorescent conjugate which would distribute uniformly within the accessible volume. Labelled bovine serum albumin, ovalbumin, or denatured proteins have been used in controls for cytoplasmic components^{13,15,17,20} and fluorescent lipid probes⁴¹ can be used in studies on membrane-associated factors. It is imperative that co-incorporation of the experimental conjugate and the control conjugate labelled with a second probe be accomplished in the same cell when the cell shape is irregular¹³. Adequate comparisons cannot be performed in separate cells when they have irregular geometries. However, separate cells can be used when the cell geometry is reasonably constant and simple¹⁷.

The local environment around the fluorescent conjugate could also affect the fluorescence intensity by altering the quantum yield or fluorescence spectral properties. Local variations in ionic parameters such as pH and ionic strength as well as binding of other molecules to the conjugates could change the fluorescence parameters^{6,7}. The sensitivities of fluorophores can either be utilised in characterising microenvironments or they must be controlled when other parameters are under investigation. Controls for the ionic environments can be performed by comparing the fluorescence of the experimental conjugates with the fluorescence of separate cells containing nonspecific molecules (such as ovalbumin) labelled with the same fluorophore¹⁷, while controls for the effect of specific molecular interactions require the extrapolation of data from solution spectroscopic studies¹⁶.

Knowledge of the optical properties of the microscope is important for interpreting fluorescence images and quantitating local fluorescence properties. The characterisation of the fluorescence image *in vivo* depends on the depth of focus of the microscope. A large depth of focus in relation to cell thickness

optimises the formation of an in focus image of the whole cell, while a small depth of focus yields an optical section of the cell. The latter condition would require several changes in focus to reconstruct the three-dimensional image of the cell. Quantitative measurements of fluorescence intensity are further affected by the extent of selecting light from specific planes of the specimen. This latter problem has been solved by using a combination of laser illumination and diaphragms placed in the image plane³⁹.

The local concentration of the fluorescent conjugates also affects the fluorescence intensity measured or visualised in different parts of cells. The distribution of the conjugates can be determined by applying a combination of the controls used for accessible volume and environmental sensitivity.

Nonperturbing indicators of physiological processes

Nonperturbing fluorescent conjugates can be used as indicators of mobility on, within, or between cells. Fluorescence indicators of normal cell-surface mobility have been studied for many years. In an early study Jeon and Bell⁴⁴ used fluorescently labelled antibodies to the cell surface of *Amoeba proteus* and a basic protein derived from a papain preparation to label the cell surface. Results based on a double labelling technique suggested that part of the cell surface could move independently of the lipid portion of the membrane.

In an elegant study, rhodamine-labelled peptides of different sizes have been microinjected into living cells coupled by gap junctions to probe the permeability and exclusion limit of the gap junction channels^{45,46}. Molecules of molecular weight up to 1,200 pass through the channels of *Chironomus* salivary gland cells which indicates that the channels are ~1.0–1.4 nm in diameter. The selectivities of the various channels have been further characterised by varying the total charge of the labelled peptides⁴⁵. For example, several mammalian cell channels can discriminate between 1–3 negative charges on the peptide backbones. The larger electronegativity inhibits passage. In addition, the permeability of multiple components has been directly compared by using mixtures of conjugates prepared with different probes^{45,46}. A control for peptide degradation has also been reported.

Stacey and Allfrey used a similar technique and microinjected a wide variety of rhodamine conjugated proteins into living HeLa cells⁴⁷ to study the process of autophagy (Fig. 1). The segregation of microinjected proteins into autophagic vacuoles exhibited a high degree of selectivity with higher molecular weight proteins turning over faster than low molecular weight proteins. One protein (haemoglobin) never became autophagocytosed. The results with fluorescently conjugated proteins were verified with immunofluorescent and autoradiographic techniques.

More sophisticated applications of nonperturbing fluorescent conjugates involve the use of environmentally sensitive fluorophores and spectral analyses to probe intracellular environments. Fluorescein-labelled ovalbumin has been used to measure the cytoplasmic pH of single cells⁴⁸ based on the observation that the excitation spectra of fluorescein is highly pH dependent⁴⁹. The pH is measured by determining the ratio of fluorescence emission intensity when the cells are excited at two different wavelengths. The effects of local pathlength are normalised since the ratio of intensities is determined. This fluorescence technique of measuring pH is less perturbative and permits better spatial resolution than standard microelectrode methods.

When applying fluorescent conjugates as indicators, the biological effects of the conjugates and the experimental procedures should be carefully examined to make sure that they are actually nonperturbing. The possible degradation of the conjugates inside the cells should also be considered, especially when the conjugates are used as size indicators^{46,47}. This latter problem could be checked more critically in the future by isolating the labelled proteins from the cells after incorporation

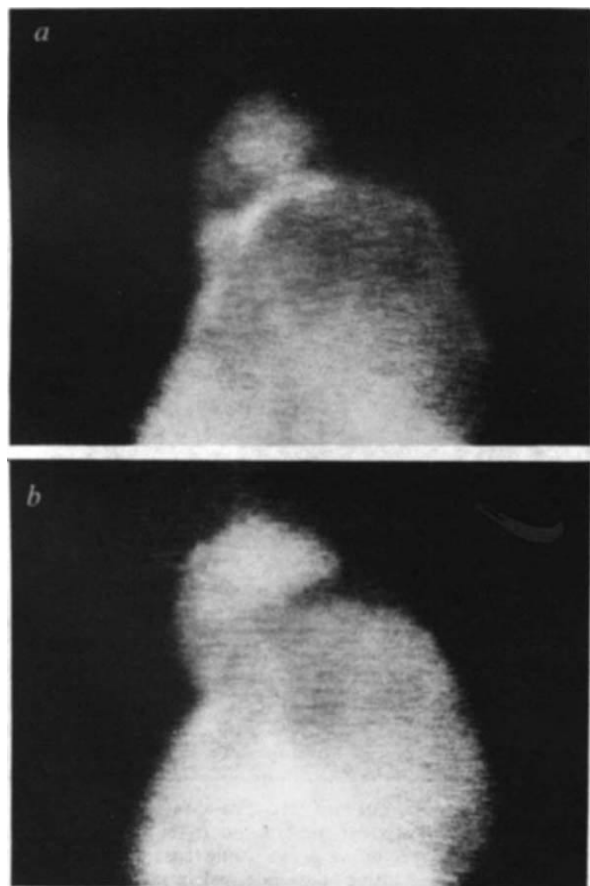


Fig. 4 Fluorescence of 5-iodoacetamidofluorescein-labelled actin (a) and lissamine rhodamine B sulphonyl chloride labelled ovalbumin (b) in the same specimen of *C. carolinensis*. Actin specific fibrils can be detected in the plasmagel sheets at the tips of advancing pseudopods (a). (From Taylor *et al.*¹³.)

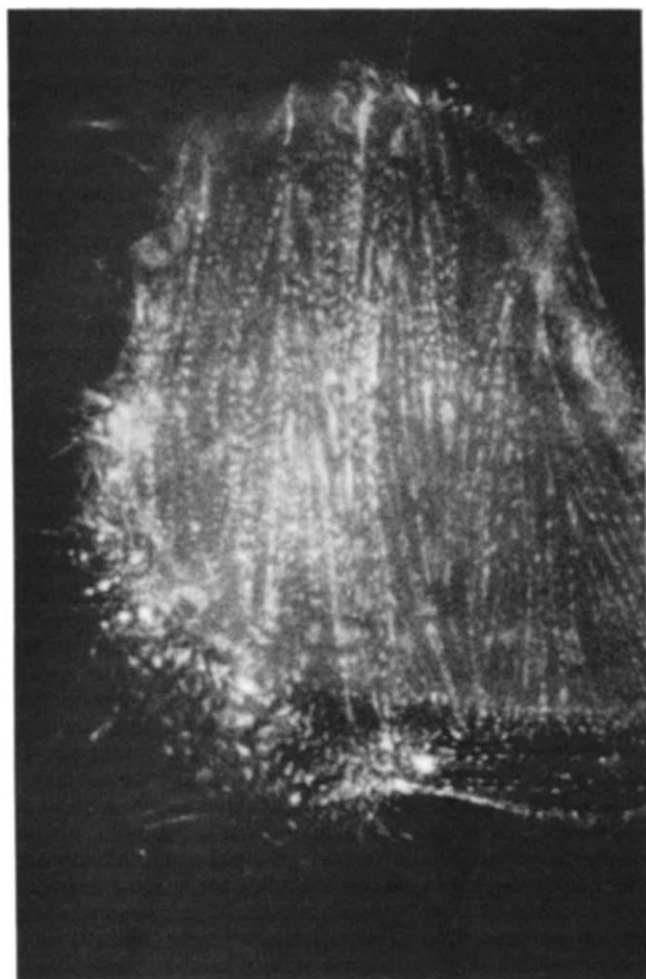


Fig. 5 Tetramethylrhodamine isothiocyanate labelled α -actinin incorporated into a living gerbil fibroblast cell. (From Feramisco²⁰.)

by fusion techniques. Furthermore, when spectral characteristics are used as indicators of local environments such as pH, the possible effects of other environmental factors must be ruled out. In all cases, calibration must be performed *in situ*⁴⁸.

Biologically active agents

Biologically active agents which can stimulate, alter or block cell functions include lectins, antibodies, drugs and toxins. These agents are used in probing cell structures and functions. Many of these agents can be fluorescently labelled, permitting detailed investigations on the distribution and mobility of binding sites. The relationship between distribution and biological effects of the labelled agents can also be studied.

The plant lectin, concanavalin A (Con A), has been used as a model ligand in investigations on receptor-ligand complexes⁵⁰⁻⁵⁷. Fluorescently labelled Con A has facilitated studies on the mobility of Con A receptors, the ultimate fate of the ligand and the relationship between receptor mobility and Con A stimulated cellular events⁵¹⁻⁵⁷. It must be emphasised that Con A has many complex effects on cells⁵⁷ and it is difficult to define the complete molecular sequences of events. Similar but more specific experiments have also been performed with antibodies prepared against various cell-surface receptors⁵⁶⁻⁵⁸. These cell-surface associated fluorescent conjugates have been used to measure the mobility of membrane components using several different approaches. The simplest method is the observation of large cells such as muscle fibres⁵⁹ in the fluorescence microscope. Small labelled spots on the cells can be detected when spread over relatively large distances. A second approach involves fusing cells containing different surface fluorescent

conjugates and following the redistribution of the labelled conjugates in the microscope⁶⁰. Poo and colleagues⁶¹ have also studied the accumulation of labelled surface receptors in a uniform electric field applied across the surfaces of cells. Finally, several investigators have used the photobleaching techniques discussed above.

Some drugs can also be fluorescently labelled while maintaining activity. The purpose of labelling the drugs is to study the correlation between the cellular effects and the localisation within the cell. Examples are the tubulin-binding drug colchicine⁶², and the actin-binding cyclic peptides phalloidin¹⁹ and phalloidin⁶³. At the proper concentration, colchicine can cause microtubule depolymerisation and phalloidin and phalloidin can cause actin polymerisation and stabilisation (see ref. 64 for references). Fluorescent conjugates of these drugs have been used to localise pools of tubulin⁶² and F-actin^{19,63} respectively in fixed or extracted cells (Fig. 2). However, it is not yet clear whether these drugs have simply identified pools of tubulin and F-actin or have also altered the normal organisation of the target proteins.

Functional cellular components (molecular cytochemistry)

Molecular cytochemistry¹⁵ has been defined as the re-incorporation of functional cellular components into or onto living cells following purification, fluorescent labelling and assaying function *in vitro*. The experimental protocol is shown in Fig. 3 using actin as a model. The feasibility of molecular cytochemistry has been initially demonstrated using actin labelled with a non-destructive probe, 5-iodoacetamido-fluorescein (IAF). The labelled actin has been proven to be functional in the purified form, in cell-free extracts, and in single-cell models^{13,15-17}. Microinjection of this fluorescent

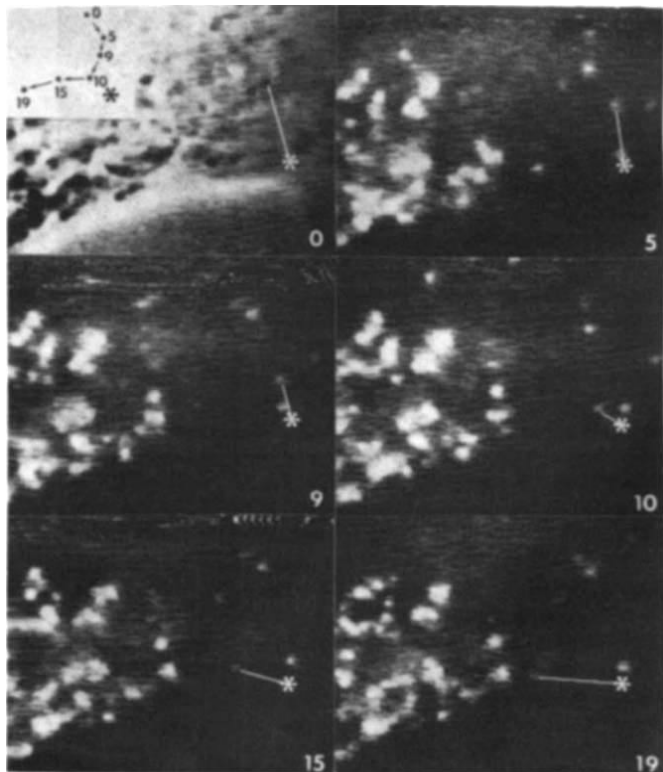


Fig. 6 Saltatory motion of fluorescent vesicles in 3T3 cells 24 h after incubation with rhodamine-labelled α_2 -macroglobulin. A time lapse video tape recording at a 9:1 time lapse ratio was made of a cell with vigorous intracytoplasmic vesicle saltatory motion. The numbers in the lower right corner of each single frame image represent the real time in seconds after the beginning of the sequence. A phase image is presented at zero time followed by fluorescence images. The asterisk represents an arbitrary non-moving reference point. The inset summarises the motion of this single fluorescent vesicle. (From Willingham and Pastan¹².)

actin analogue into living cells has provided a direct way of visualising actin-containing structures and following the changes in actin distribution during cellular processes such as fertilisation of eggs¹⁷, cytokinesis¹⁷, amoeboid movement^{13,15}, pinocytosis¹³, and Con A capping¹³. Extensive controls *in vitro* and *in vivo* have made the interpretation of the fluorescent images possible^{13,15-17} (Fig. 4). This approach has recently been applied to other cells^{20,21} and other contractile proteins²⁰ (Fig. 5).

The use of fluorescent conjugates specific for receptors in the cell surface has permitted the characterisation of the kinetics of the distribution and the ultimate fate of many ligands. These investigations have included studies on α_2 -macroglobulin^{12,65,66}, epidermal growth factor⁶⁶⁻⁶⁸, insulin⁶⁶⁻⁶⁹, a chemotactic peptide⁷⁰, low-density lipoproteins⁷¹, and acetylcholine receptors⁷¹⁻⁷⁴. All of these fluorescent ligands initially label the cell surface uniformly, but at least some of the ligand-receptor complexes aggregate into patches within a few minutes. Most of the fluorescent conjugates eventually become internalised^{69,75}. The role of internalisation has not yet been defined in detail. The redistribution of some of these ligand-receptor complexes in the plane of the membrane has been quantitated using the fluorescence photobleaching techniques.

Proper use of molecular cytochemistry demands the application of various biological controls in addition to the analysis of physical considerations discussed above. The functional activity of the conjugates must be carefully demonstrated. It requires that: (1) the labelling reaction does not abolish the normal biochemical activity of the substrate, (2) the conjugates have access to the intracellular domains where the function is performed, and (3) the conjugates are not rapidly degraded *in vivo*. The biochemical activities can be readily tested using *in vitro* assays, cell-free extracts or lysed cell models^{13,16}. The use of site-specific probes would provide functionally homogeneous conjugates and would yield unequivocal results with *in vitro* assays¹⁶. The studies with labelled actin have yielded the most definitive biological controls^{13,15-17} to date.

The function inside living cells is much more difficult to assay. For IAF-labelled actin mentioned above, well characterised cellular responses such as cortical wound healing, can be used to test the functionality *in situ*¹³. Furthermore, a comparison of the fluorescent images of biologically active conjugates with non-functional conjugate controls would also indicate the formation of structures related to biological functions^{13,15,17}. Note that the absence of fluorescence from a cellular domain, such as the nucleus^{13,17}, does not necessarily imply that the endogenous component is not present. Negative results could be due to physical exclusion of the conjugate, slow turnover time for the incorporation into specific structures, as well as the absence of a cellular component from a particular region. Therefore, negative results must also be interpreted with caution.

Future prospects

This new approach to cell biology is likely to develop extensively both technically and in its range of biological applications. At the technical level, improvements in the hardware and software applied to intensified image recording and spectral analysis should permit sophisticated manipulations of the experimental data. Measurements of fluorescence polarisation^{14,76,77}, resonance energy transfer^{60,78-80} and fluorescence lifetime could be performed and factors such as accessible volume could be corrected automatically. Detailed molecular information including microviscosity, local polarity, rotational freedom and formation of supramolecular structures could be determined.

The application of this new technique is expected to extend to many different areas of cell biology, through the use of fluorescent conjugates of nonperturbing indicators, biologically active agents and functional cellular components. Components binding specific ions or ligands could be labelled with environmentally sensitive probes and used as indicators of intracellular environments. One such possibility would be the calcium binding protein calmodulin⁸¹. Some drug studies previously relying on radioactive derivatives could be performed with fluorescent

conjugates which would allow the direct observation of uptake, distribution and turnover. Furthermore, the new techniques of blocking cell functions with specific antibodies^{82,83} or modified cellular components⁸⁴ can be combined with the present approaches yielding a more powerful technique. The most dramatic advances will probably be seen in the area of molecular cytochemistry using functional conjugates of proteins, lipids, nucleic acids, carbohydrates and even whole organelles. Careful use of molecular cytochemistry is expected to bring new insights into areas as diverse as cell motility, virus-cell interactions, nuclear-cytoplasmic interactions, cell-cell interactions in tissues, axonal transport, gene expression, and assembly of organelles at both morphological and molecular levels. A direct connection between cell physiology, biochemistry and ultrastructure would then become a reality.

We thank J. Heiple, V. Fowler, B. Luna, L. Tanasugarn, L. Simons, E. Haas and M. Rizzo for helpful discussions, and L. Stryer and J. Yguerabide for reading the manuscript. Valuable collaboration with B. Ware and F. Lanni on fluorescence photobleaching is also acknowledged. Some of the research reported here was supported by NSF grant PCM-7822499 and NIH grant AM 18111.

- Chambers, R. & Chambers, E. L. *Explorations into the Nature of the Living Cell* (Harvard University Press, Cambridge, Massachusetts, 1961).
- Lorch, I. J. in *The Biology of Amoeba* (ed. Jeon, K. W.) 1-36 (Academic, New York, 1973).
- Jeon, K. W., Lorch, I. J. & Danielli, J. F. *Science* **167**, 1626-1627 (1976).
- Hiramoto, Y. *Exp. Cell Res.* **27**, 416-426 (1962).
- Nichols, K. M. & Rikmenspoel, R. J. *Cell* **29**, 233-247 (1978).
- Guilbault, G. G. *Practical Fluorescence. Theory, Methods and Techniques* (Dekker, New York, 1973).
- Fluorescence Spectroscopy (eds Pesce, A. J., Rosen, C. G. & Pasby, T. L.) (Dekker, New York, 1971).
- Thaer, A. A. & Sernetz, M. (eds) *Fluorescence Techniques in Cell Biology* (Springer, Berlin, 1973).
- Reynolds, G. T. *Quant. Rev. Biophys.* **5**, 295-347 (1972).
- Reynolds, G. T. & Taylor, D. L. *BioScience* (in the press).
- Sedlacek, H. H., Gundlach, H. & Ax, W. *Behring. Inst. Mitt.* **59**, 64-70 (1976).
- Willingham, M. C. & Pastan, I. *Cell* **13**, 501-507 (1977).
- Taylor, D. L., Wang, Yu-Li & Heiple, J. J. *Cell Biol.* (submitted).
- Sengbusch, G. V. & Thaer, A. in *Fluorescence Techniques in Cell Biology* (eds Thaer, A. A. & Sernetz, M.) 31-39 (Springer, Berlin, 1973).
- Taylor, D. L. & Wang, Y.-L. *Proc. natn. Acad. Sci. U.S.A.* **75**, 857-861 (1978).
- Wang, Yu-Li & Taylor, D. L. *J. Histochem. Cytochem.* (submitted).
- Wang, Y.-L. & Taylor, D. L. *J. Cell Biol.* **82**, 672-679 (1979).
- Cherry, R. J., Cognoli, A., Opplinger, M., Schneider, G. & Semenza, G. *Biochemistry* **15**, 3653-3656 (1976).
- Barak, L. S., Yocum, R. R., Nothnagel, E. A. & Webb, W. W. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
- Feramisco, J. R. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3967-3971 (1979).
- Kreis, T. E., Winterhalter, K. H. & Birchmeier, W. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3814-3818 (1979).
- Waggoner, A. S. A. *Rev. Biophys. Bioengng* **8**, 47-68 (1979).
- Diacumakos, E. G. *Meth. Cell Biol.* **7**, 288-311 (1978).
- Blinks, J. R. *et al. Meth. Enzym.* **58**, 292-328 (1978).
- Rose, B. & Lowenstein, W. R. *J. Membrane Biol.* **28**, 87-199 (1976).
- Schlegel, R. A. & Richsteiner, M. C. *Meth. Cell Biol.* **20**, 341-354 (1978).
- Tyrell, D. A., Heath, T. D., Colley, C. M. & Ryman, B. E. *Biochim. biophys. Acta* **457**, 259-302 (1976).
- Gregoriadis, G. *New Engl. J. Med.* **295**, 704-770 (1976).
- Yamaizumi, M., Uchida, T., Mekada, E. & Okada, Y. *Cell* **18**, 1009-1014 (1979).
- Williamson, R. E. *J. Cell Sci.* **17**, 655-668 (1975).
- Miller, M. R., Castellot, J. J. Jr & Pardee, A. B. *Exp. Cell Res.* **120**, 421-425 (1979).
- Kohen, E., Kohen, C. & Thorell, B. *Exp. Cell Res.* **81**, 477-482 (1973).
- West, S. S. in *Physical Techniques in Biological Research* Vol. 3C (ed. Pollister, A. W.) 253-321 (Academic, New York, 1969).
- Ploem, J. S., Starke, J. A., De, Bonnet, J. & Wasmund, H. J. *Histochem. Cytochem.* **22**, 668-677 (1974).
- Malmstadt, H. V., Franklin, M. L. & Horlick, G. *Analyt. Chem.* **44**, 63A-76A (1972).
- Wreford, N. G. M. & Schafield, G. G. *J. Microsc.* **103**, 127-130 (1974).
- Rost, F. W. D. & Pearce, A. G. E. *J. Microsc.* **94**, 93-105 (1971).
- Cova, S., Prena, G. & Mazzini, G. *Histochem. J.* **279**, 299 (1974).
- Axelrod, D., Koppel, D. E., Schlessinger, J., Elson, E. & Webb, W. W. *Biophys. J.* **16**, 1055-1060 (1976).
- Elson, H. & Yguerabide, J. *J. supramolec. Struct.* **12** (in the press).
- Smith, B. A. & McConnell, H. M. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2759-2763 (1978).
- Koppel, D. E. *Biophys. J.* **28**, 281-292 (1979).
- Sheetz, M. P. & Koppel, D. E. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3314-3317 (1979).
- Jeon, K. W. & Bell, L. G. E. *Exp. Cell Res.* **33**, 531-539 (1964).
- Flagg-Newton, J., Simpson, I. & Loewenstein, W. R. *Science* **205**, 404-407 (1979).
- Simpson, I., Rose, B. & Loewenstein, W. R. *Science* **195**, 294-296 (1977).
- Stacey, D. W. & Allfrey, V. G. *J. Cell Biol.* **75**, 807-817 (1977).
- Heiple, J. & Taylor, D. L. *J. Cell Biol.* (submitted).
- Ohkuma, S. & Poole, B. *Proc. natn. Acad. Sci. U.S.A.* **75**, 3327-3331 (1978).
- Bittiger, H. & Schnebli, H. P. (eds) *Concanavalin A as a Tool* (Wiley, New York, 1976).
- Schlessinger, J. *et al. Proc. natn. Acad. Sci. U.S.A.* **73**, 2407-2413 (1976).
- Yahara, I. & Edelman, G. M. *Exp. Cell Res.* **81**, 143-155 (1973).
- Ryan, G., Borysenko, J. Z. & Karnovsky, M. J. *J. Cell Biol.* **62**, 351-370 (1974).
- Oliver, J. M., Ukena, T. E. & Berlin, R. D. *Proc. natn. Acad. Sci. U.S.A.* **71**, 394-398 (1974).
- Condeelis, J. R. *J. Cell Biol.* **80**, 751-758 (1979).
- Taylor, R. B., Duffus, P. H., Ross, M. C. & DePetrus, S. *Nature new Biol.* **233**, 225-229 (1971).

57. Schreiner, G. F. & Unanue, E. R. *Adv. Immun.* **24**, 38–165 (1976).
58. Woda, B. A., Yguerabide, J. & Feldman, J. D. *J. Immun.* **123**, 2161–2167 (1979).
59. Edidin, M. & Fambrough, D. *J. Cell Biol.* **57**, 27–53 (1973).
60. Keller, P., Person, S. & Snipes, W. J. *J. Cell Sci.* **28**, 167–177 (1977).
61. Poo, M.-m., Poo, W. J. H. & Lam, J. W. J. *J. Cell Biol.* **76**, 483–501 (1978).
62. Clark, J. & Garland, D. *J. Cell Biol.* **76**, 619–627 (1978).
63. Wulf, E., Deboen, A., Bautz, F. A., Faulstich, H. & Wieland, T. *Proc. natn. Acad. Sci. U.S.A.* **76**, 4498 (1979).
64. Taylor, D. L. & Condeelis, J. S. *Int. Rev. Cytol.* **56**, 57–144 (1979).
65. Pastan, I., Webb, W. W. & Elson, E. L. *Proc. natn. Acad. Sci. U.S.A.* **74**, 2909–2913 (1977).
66. Schlessinger, J., Schechter, Y., Willingham, M. & Pastan, I. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2659–2663 (1978).
67. Schechter, Y., Hernaez, L., Schlessinger, J. & Cuatrecasas, P. *Nature* **278**, 835–838 (1979).
68. Haigler, H., Ash, J. F., Singer, S. J. & Cohen, S. *Proc. natn. Acad. Sci. U.S.A.* **75**, 3317–3321 (1978).
69. Maxfield, F. R., Schlessinger, J., Schechter, Y., Pastan, I. & Willingham, M. C. *Cell* **14**, 805–810 (1978).
70. Niesel, J. E., Kahane, I. & Cuatrecasas, P. *Science* **205**, 1412–1414 (1979).
71. Krieger, M. et al. *J. supramolec. Struct.* **10**, 467–478 (1979).
72. Axelrod, D. et al. *Proc. natn. Acad. Sci. U.S.A.* **73**, 4594–4598 (1976).
73. Anderson, M. J. & Cohen, M. W. *J. Physiol., Lond.* **237**, 385–400 (1974).
74. Block, R. J. *J. Cell Biol.* **82**, 626–643 (1979).
75. Silverstein, S. C., Steinman, R. M. & Cohn, Z. A. *Rev. Biochem.* **46**, 669–722 (1977).
76. Axelrod, D. *Biophys. J.* **26**, 557–574 (1979).
77. Nihei, T., Mendelson, R. A. & Botts, J. *Biophys. J.* **14**, 236–242 (1974).
78. Stryer, L. *Rev. Biochem.* **47**, 819–846 (1978).
79. Wu, C. W. & Stryer, L. *Proc. natn. Acad. Sci. U.S.A.* **69**, 1104–1108 (1972).
80. Fernandez, S. M. & Berlin, R. D. *Nature* **264**, 411–415 (1976).
81. Potter, J. D. et al. in *Calcium Binding Proteins and Calcium Function* (eds Wasserman, R. H. et al.) 239–249 (North-Holland, New York, 1977).
82. Mabuchi, I. & Okuno, M. *J. Cell Biol.* **74**, 251–263 (1977).
83. Rungger, D., Rungger-Brändle, E., Chaponnier, C. & Gabbiani, G. *Nature* **282**, 320–321 (1979).
84. Meeusen, R. L. & Cande, W. Z. *J. Cell Biol.* **82**, 57–65 (1979).

ARTICLES

Variations in the thermal emission of Seyfert galaxies

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Accurate monitoring of five Seyfert galaxies demonstrates that they are all variable in the IR region. These fluctuations probably arise when variations in the nonthermal UV sources change the rate of heating of interstellar dust within the nuclei of these galaxies, as demonstrated quantitatively for III Zw 2.

AS soon as the large IR excesses of Seyfert galaxies were discovered, it was recognised that any variability of these excesses could be a vital clue to their nature, in particular as to whether they had a thermal or a nonthermal origin. Despite relatively large measurement uncertainties, early programmes produced evidence that variations do occur^{1–4}. It followed then that greater accuracy would be required to study the variations in any detail, as well as to convince sceptics of the reality of the changes observed. The large change reported recently for 3C120 in the IR⁵ seems to be exceptional, and any general monitoring programme should consider variations of 10–20%.

Recent advances in near IR detector technology make possible routine differential photometry between the brighter Seyfert galaxies and selected nearby stars with an accuracy of 1–2%. This article reports such photometry for III Zw 2, NGC1275, 3C120, NGC4151 and NGC 5548, galaxies selected because of their high degree of variability in the visible. During our observations, all five galaxies varied by many times the measurement errors. Comparison of coordinated IR and visible observations shows that the variations in the two spectral regions are not simultaneous. The behaviour of these galaxies indicates that much of the UV emission of their nuclei is absorbed by interstellar dust and is re-radiated in the IR.

New observations

Our photometry is listed in Tables 1 and 2. Most of the IR measurements were obtained on the Catalina 1.54-m (61-in) telescope, with a field of view 8.5 arc s in diameter and relative to reference areas 10 arc s to the east and west of the nucleus of the galaxy. A few measurements were made on the Steward 2.24-m (90-in), where a beam of 7.8 arc s was used with reference areas 10 arc s to the north and south of the nucleus. No systematic differences are apparent between measurements with the two telescopes.

Two photometers were used, one with a liquid nitrogen cooled InSb detector and one with a liquid helium cooled detector of the same type. Although the filters defining the spectral passbands were identical for the photometers, the lower operating temperature with the second instrument produced slight shifts in filter characteristics. The effects of these shifts

were calibrated and removed from the data by comparing measurements with both photometers in August 1978.

The tabulated errors for the IR photometry reflect only the combined scatter of statistical uncertainties and of a series of comparisons with the local standards on that night. Typically, three or four comparisons were made in each colour. The measurements have been converted to fluxes by tying the local standards in with the network established by Johnson *et al.*⁶ and applying the calibration at $J(1.25 \mu\text{m})$ of 0.0 mag = 1,770 Jy, at $H(1.6 \mu\text{m})$ 0.0 mag = 1,080 Jy, at $K(2.2 \mu\text{m})$ 0.0 mag = 680 Jy, and at $L(3.5 \mu\text{m})$ 0.0 mag = 300 Jy. We believe that the local standards are tied into the primary system with an accuracy of at least 3% (6%, for NGC5548). The absolute calibration is also subject to an uncertainty of 5–10%. Fortunately, for virtually all of our analysis only the uncertainties in the comparisons with the local standards are important.

Photoelectric measurements were obtained with a Johnson *UBVRI* photometer on the Mt Lemmon 1-m (40-in) telescope. The comparison sequences listed by Lyutyi⁷ were used except for III Zw 2, where local standards were determined as part of this programme. As with the IR observations, the tabulated errors refer only to the comparisons with the local standards. The absolute calibration is described by Johnson⁸. The measurements of III Zw 2 on 11 December 1979 were provided by Wieslaw Wisniewski.

Nature of IR variations

Tables 1 and 2 also include an average over the measurements in the J , H , K and L spectral bands for each date, normalised to 1.00 for an arbitrary date and weighted by the tabulated errors. This parameter, plotted in Fig. 1, is convenient for showing the extent of overall variability, although in some cases we have found a strong spectral dependence to the changes (note III Zw 2, discussed below). The same parameter is shown for previous measurements⁵, in the cases of III Zw 2 and 3C120, the parameter is normalised to the data of August 1978, reported in this earlier publication. As is clear from Fig. 1, all five of the galaxies show IR variability at a high degree of confidence.

The five galaxies were selected for this programme on the basis of their large amplitude optical variability. In other

Table 1 Coordinated optical and IR photometry of Seyfert galaxies

Source	Date (UT)	U	B	V	R	I	J	H	K	L	(IR)	$\delta(\text{IR})$
III Zw 2	8.27.78	3.9*	4.65†	5.63†	7.4*	7.0*	9.1†	12.2†	22.1		0.99	0.02
	1.5.79		4.88				10.1	13.3	23.2		1.05	0.02
	9.20.79	2.74†	3.13	4.17	6.0‡	5.4‡	8.8†	14.0	27.1		1.16	0.02
	12.3.79	3.30†	3.73	4.48†		7.5‡	8.6	13.7	26.3		1.13	0.02
NGC1275	8.27.78	7.4†	15.1	23.7	35*	53†	55	69	93		1.00	—
	11.19.78	6.0*	13.4	21.2	32*	43†	37	50	67		0.70	0.01
	9.20.79	6.7†	12.9	20.9†			40.5	51	67†		0.73	0.01
	12.5.79		12.5	20.5	34*	45†	35	46	63		0.65	0.01
3C120	8.27.78	1.40*	1.85†	3.80†			10.8†	15.0	23.6		1.07	0.02
	11.19.78		2.35†	4.0*			10.7	14.2	24.1		1.07	0.02
	9.20.79		2.2†	4.4*			10.0†	17.1	28.3		1.18	0.02
NGC4151	4.3.78	39†	48	65	107	122	127	176	227	371	1.00	—
	3.7.79	16.1†	29.6	47	87	97	86	116†	162		0.68	0.01
	5.11.79	31.2	44.8	63	105	118			152		0.67	0.02
NGC5548	4.3.78	11.4†	14.3	21.0	31.8	36.1	35.0	42.2	57.1	90	1.00	—
	3.7.79	5.8†	8.3†	13.4			24.2	31.4	42.5		0.72	0.02

0 < errors < 2% unless otherwise indicated; the error in (IR) (the normalised IR brightness) is indicated as $\delta(\text{IR})$ and is based on the inverse square weighting of the changes in the IR bands.

* 4% < errors ≤ 6%. † 2% < errors ≤ 4%. ‡ 6% < errors ≤ 10%. Fluxes in mJy = $10^{-29} \text{ W m}^{-2} \text{ Hz}^{-1}$. 23 arc s aperture for *UBVRI*, 8.5 arc s aperture for *JHKL*.

respects, they represent a very broad range of spectral properties and luminosities. As they all varied, we believe that virtually all Seyferts that vary in the visible also vary in the IR.

The simplest explanation for variations in both spectral regions would be that a single, nonthermal source dominates the output of these galaxies from the visible through the IR. However, the variations are not simultaneous between the visible and IR, and in some cases they even occur in the opposite sense in the two spectral regions. This behaviour differs dramatically from that of nonthermal sources such as BL Lac type objects⁹. We therefore will consider models in which different sources dominate the IR and optical spectra.

For these models, the spectra of the nuclei of the galaxies can be divided into four components: (1) a rapidly fluctuating, presumably nonthermal, source that dominates the UV continuum; (2) continuum and line emission by ionised gas, the intensity of which can be expected to vary much more slowly and with smaller amplitude than the UV continuum; (3) the direct output of stars; and (4) the IR excess. Coordinated optical to IR photometry can be combined with spectral photometry to determine the spectra of the UV and IR source components.

Spectra of IR sources

Separation of the UV and IR components of the nuclear source in III Zw 2 should be relatively straightforward, as the nonthermal (optical-UV) continuum is presumably of much higher luminosity than any stellar contribution (the source is sometimes classified as a QSO because of its compact appearance on plates). We assume that the spectra of the continuum components do not change during variations and that the continuum flux at B is entirely from the nonthermal source. The first of these assumptions is suggested by our photometry but needs to be tested by additional observation; a justification for the second follows from our calculations below. With these assumptions, it is possible to estimate the relative contributions of the nonthermal and IR sources at J, using the fact that the variation at J is intermediate between that at B and K, and thus that J includes contributions from both the nonthermal and the IR sources. A crude estimate would assume that the nonthermal source contributes negligibly to K. This estimate can be refined in an iterative way by extrapolating the nonthermal continuum as a power law from B and J to K, determining the variation of the IR source alone at K, and repeating the separation at J. For 1 May 1979 one finds that the nonthermal fluxes at J, H, and K are respectively 7.3, 8.0 and 9.0 mJy, in reasonably good agreement with a simple extrapolation of the optical-UV continuum¹⁰. The power law fit to the B and J fluxes has a slope of -0.4. The IR

source produces fluxes at J, H and K of 2.8, 5.3 and 14.2 mJy, respectively, and a power law fit from J to K would have an index of ~ -3 .

The very rapid fall in the spectrum of the IR source towards shorter wavelengths was already indicated for several other Seyfert galaxies by spectral inflections near $1 \mu\text{m}$ (ref. 11) and, for NGC1275, 4151, 5548 and 7469, by an approximate separation of the spectrum into stellar, variable UV, and IR components.¹¹ The separation of source components by accurate, coordinated measurements of variability from the UV into the IR should be more reliable than these other two methods. However, for 3C120, NGC1275 and NGC5548 our data are not yet sufficiently extensive for such an analysis. The variability of NGC4151 will be discussed in detail elsewhere.

Role of dust in Seyfert galaxy nuclei

The slope of the spectrum of the IR component in III Zw 2 corresponds to a colour temperature between 1.25 and $2.2 \mu\text{m}$ of 1,500 K, corresponding closely to the maximum temperature at which interstellar grains can survive. If the near IR flux is

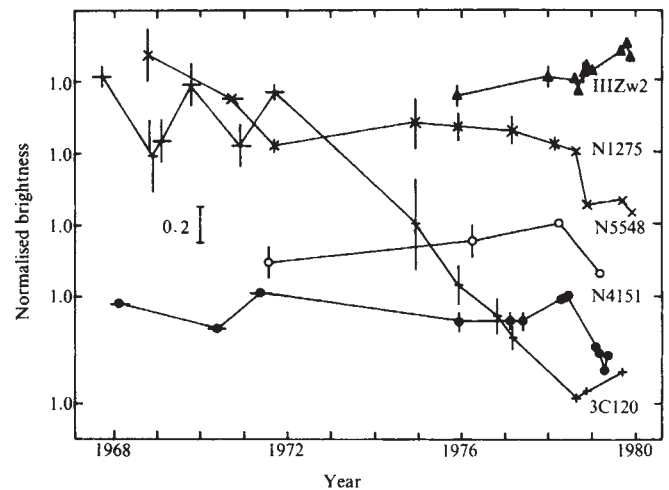


Fig. 1 IR Variability of five Seyfert galaxies. Data reported here have been combined with those summarised in ref. 5. The spectrally averaged IR brightness for each galaxy has been normalised to 1.0 near the middle of 1978. The bar at the beginning of 1970 shows the amplitude of a variation of 20% of the normalisation value. When measurement errors exceed the sizes of the symbols, they are indicated with vertical bars. When measurements over an extended period have been combined into a single point, the total period of measurement is shown with a horizontal bar.

radiated by hot, interstellar grains certain limitations can be placed on the size of the emitting region. Assuming the distance is as indicated by a cosmological interpretation of the redshift of $z = 0.089$ with $H_0 = 55 \text{ km s}^{-1} \text{ Mpc}^{-1}$, the radius of a 1,500 K blackbody that can generate the observed near IR fluxes is $\sim 0.2 \text{ pc}$. If the central UV source has a luminosity of $\sim 10^{12} L_\odot$ (see below) and the absorptivity of the grains is neutral between the UV and IR, the grains will reach an equilibrium temperature of 1,500 K at a distance of $\sim 0.2 \text{ pc}$ from the central source. The IR source must be somewhat larger than either of these estimates, as the bright optical and UV flux from III Zw 2 demonstrates that the source is optically thin (at least towards the Earth) and most plausible dust constituents have greater UV than IR absorptivity, raising the equilibrium temperature for a given distance from the central source. However, the dependences on radius are sufficiently steep that the size should be close to the lower limits estimated above: a size of $\sim 0.6 \text{ pc}$ would require an optical depth of only 10% and UV absorptivity 16 times that in the IR. A thermally re-radiating source 0.6 pc in radius would smooth out all variations of the UV flux and delay them with a time constant of $\sim 2 \text{ yr}$. This estimate agrees well with the brightening of III Zw 2 in the IR in September 1979, $\sim 2 \text{ yr}$ after the onset of a powerful optical-UV and radio outburst¹². Further observations in the IR can test this association.

There is already evidence that most Type 2 and those Type 1 Seyfert galaxies with steeply rising IR spectra, such as NGC4151 and NGC5548, emit thermally in the IR¹¹. Among Type 1 Seyfert galaxies, III Zw 2 has one of the most slowly rising spectra into the IR. From its behaviour, re-radiation in the IR by dust is an important component of its spectrum also.

The luminosity of the IR source in III Zw 2 is $\sim 4 \times 10^{11} L_\odot$ (ref. 11). From the strength of $H\alpha$ ¹³, the luminosity in the Lyman continuum must be $\sim 2 \times 10^{11} L_\odot$. If the nonthermal continuum can be represented by a power law of index -0.4 from $1 \mu\text{m}$ to the Lyman limit, the luminosity over this spectral range is $\sim 6 \times 10^{11} L_\odot$. Therefore, a significant proportion of the UV continuum of the nonthermal source is absorbed by dust and re-radiated in the IR. The ratio of $H\alpha$ to IR luminosity for III Zw 2 is among the largest for Seyfert galaxies; for most of these

Table 2 Additional IR photometry

Source	Date (UT)	J	H	K	L	(IR)	$\delta(\text{IR})$
III Zw 2	9.10.78	8.6	11.1†	21.4		0.94	0.02
	11.2.78			23.2*		1.04	0.06
	11.19.78	10.8	13.8	23.9		1.08	0.02
	11.12.79	8.9	14.8	28.3	46‡	1.20	0.02
NGC4151	4.24.78	119†	173	231	361	0.97	0.02
	5.22.78		165†	229	392	1.00	0.02
	5.30.78	118†	168	227		0.96	0.02
	6.18.78	126	173†	238		1.00	0.02
	2.9.79	92	124†	166		0.72	0.01
	4.13.79	81‡	104†	136		0.60	0.01
	4.19.79			128		0.56	0.02

Fluxes, aperture, and errors as in Table 1. 0 < errors < 2% unless otherwise indicated.

* 4% < errors $\leq$ 6%. † 2% < errors $\leq$ 4%. ‡ 6% < errors $\leq$ 10%.

sources the role of dust will be relatively larger than in III Zw 2. From the time scale of the IR variations for all five galaxies we have studied, at least part of this dust lies within 0.1–0.6 pc from the nonthermal sources, depending on the galaxy. The dust therefore lies within or at the outer boundary of the region responsible for the broad emission lines in the Type 1 galaxies.

Conclusions

From accurate monitoring of the near IR fluxes of five Seyfert galaxies, we conclude:

Most or all Seyfert galaxies that vary in the optical-UV also vary in the near IR.

The IR variations are not simultaneous with the optical-UV ones, but have the characteristics of dust reradiating the absorbed nuclear UV continuum.

From the time scale of the IR variability, the dust lies near the gas producing the broad emission lines in Type 1 Seyferts.

We thank G. V. Coyne and W. Z. Wisniewski for instruction in the techniques of photoelectric photometry. This work was supported by the NSF. G. H. R. is an Alfred P. Sloan fellow.

Received 14 December 1979; accepted 13 February 1980.

1. Pacholczyk, A. G. *Astrophys. J.* **163**, 449–454 (1971).
2. Penston, M. V. *et al. Mon. Not. R. astr. Soc.* **169**, 357–393 (1974).
3. O'Dell, S. L., Puschell, J. J., Stein, W. A. & Warner, J. W. *Astrophys. J. Suppl.* **38**, 267–286 (1978).
4. Glass, I. S. *Mon. Not. R. astr. Soc.* **186**, 29P–33P (1979).
5. Rieke, G. H. & Lebofsky, M. J. *Astrophys. J.* **227**, 710–713 (1979).

6. Johnson, H. L., Mitchell, R. I., Iriarte, B. & Wisniewski, W. Z. *Commun. Lunar planet. Lab. Univ. Ariz.* No. 63 (1966).
7. Lyutyi, V. M. *Soviet. Astr.* **16**, 763–773 (1973).
8. Johnson, H. L. *A. Rev. Astr. Astrophys.* **4**, 193–206 (1966).
9. Rieke, G. H. & Lebofsky, M. J. *A. Rev. Astr. Astrophys.* **17**, 477–511 (1979).
10. Neugebauer, G., Oke, J. B., Becklin, E. E. & Matthews, K. *Astrophys. J.* **230**, 79–94 (1979).
11. Rieke, G. H. *Astrophys. J.* **226**, 550–558 (1978).
12. Schnopper, H. W. *et al. Astrophys. J. Lett.* **222**, L91–L94 (1978).
13. de Bruyn, A. G. & Sargent, W. L. W. *Astr. J.* **83**, 1257–1292 (1978).

The representation of colours in the cerebral cortex

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New insights into how colour is represented in the cerebral cortex and what variables govern the responses of single cortical colour-coded cells have been gained by the discovery of specific visual cortical areas rich in colour-coded cells.

OUR ability to see an almost infinite variety of colours depends on the presence, in the retina, of only a limited number of receptor types. That the number must be limited was first proposed by Thomas Young. He wrote¹, "Now as it is almost impossible to conceive each sensitive point on the retina to contain an infinite number of particles, each capable of vibrating in perfect unison with every possible undulation, it becomes necessary to suppose the number limited, for instance to the

three principal colours, red, yellow and blue". The identification of three cone pigments in the primate retina^{2–5}, each with a broad response curve, but each having a maximal sensitivity to a different part of the visible spectrum, was a striking confirmation of Young's theory. In its simplest form, this theory supposed that the colour of each 'point' in the field of view is determined by the relative responses of the three cone pigments at a corresponding 'point' in the retina and that this relative response is determined

by the amount of light in each waveband coming from that 'point'. Most colour scientists since Young have, however, assigned a role to more central nervous interactions in accounting for colour perception, and especially for colour constancy^{7,8}. This refers to the persistence of the colour of objects or of surfaces when viewed in lights of different spectral composition, such as daylight and light produced by a tungsten filament bulb. It cannot be explained in terms of energy-wavelength relationships. Other explanations have therefore been sought and, in most, the cerebral cortex has been given a dominant, but neurophysiologically a vague and ill-defined, role. Helmholtz thought learning and judgement to be critical^{6,9}. Hering considered memory to be important¹⁰. Other factors, such as adaptation, have also been suggested⁸. Land^{11,12} in his retinex theory, has sought to explain colour vision (including colour constancy) in computational terms which are essentially independent of energy, surroundings, adaptation, contrast and all psychological factors while being critically dependent on the cortex.

What role, then, does the cortex have in colour perception and how are colours represented there? We decided to explore the responses of single colour-coded cells in the cortex of the rhesus monkey, an animal close to man, with the hope of gaining some insight into these questions.

Wavelength sensitivities of colour-coded cells in the cortex

Anatomical^{13,14} and functional¹⁵⁻¹⁷ studies of rhesus monkey prestriate visual cortex have shown that the different areas within it have remarkably different populations of functional cells, thus leading to the theory of functional specialisation in the visual cortex^{17,18}. One such specialisation occurs in the fourth visual areas which, unlike other prestriate areas, are rich in colour-coded cells¹⁵⁻¹⁹. Since the fourth visual areas are the highest cortical areas to which the analysis of colour has been traced, both anatomically¹⁴ and physiologically^{16,19}, we began by studying the responses and wavelength sensitivities of cells there. Chief among the properties of these areas is the relatively large size of the cells' receptive field^{16,19} (when compared to field sizes at equivalent eccentricities in the striate cortex) and the concentration of field positions in the central 20-30° of the retina. One of the most striking features is the absence of a discernible retinotopic organisation such as that found in the striate cortex²⁰, since field positions in long oblique penetrations move in an unpredictable manner which bears no obvious relation to retinal topography.

Using conventional anatomical and electrophysiological techniques¹⁸, single cells in the fourth visual areas were isolated in the anaesthetised monkey and their receptive fields plotted. Their wavelength response curves (action spectra) were determined by establishing the threshold response at different wavelengths¹⁶. Figure 1 shows some representative narrow-band action spectra. It is evident that cells in V4 can be selective to narrow parts of the visible spectrum. Just how narrow these are is shown in Fig. 2. This is a plot of peak sensitivity against wavelength. Throughout most of the spectrum, bandwidths range from 10 to 50 nm, with many having a bandwidth at half maximum of between 10 and 20 nm. Thus the response of cells in V4 can be said to approximate more closely what may be termed responses to individual colours, or hues, than anything seen in more antecedent parts of the visual system^{21,22}.

One can now go further and ask whether cortical colour representation limits the peak sensitivities of cells to certain parts of the visible spectrum, as in the retina. To rephrase Young¹, "is it necessary to limit the peak sensitivities, for instance to the three principal colours, red, yellow and blue". Figure 3 shows the answer to be no. Between them, the peak sensitivities of these narrow band cells cover almost the entire visible spectrum. Superimposed on this wide distribution, however, is a clustering, most cells having their peak sensitivities at 480 nm (blue), 500 nm (green) and 620 nm (orange-red). Hence these are the regions of the spectrum most strongly represented in the cortex. Purple, an extraspectral colour, is also

strongly represented. Finally, it is noteworthy that, as in the striate cortex²³, no cells have peak sensitivities in the psychophysically least saturated part of the visible spectrum, that is in the 560-570 nm region²⁴, roughly where the long-wave pigment has its maximal absorption. Why this should be so is not clear. With further experiments, cells with peak sensitivities in this region may be found. So far, however, it seems that the cortical representation of this part of the spectrum is poor.

Mapping of colours in the cortex

Given such narrow band cells and given the clustering of peak sensitivities in the blue, green, purple and red, it is interesting to ask how these colours are mapped in the cortex. From micro-mapping experiments, there seems little doubt that cells with particular colour preferences are grouped together in the cortex of V4^{16,19}. This is especially well seen in perpendicular electrode

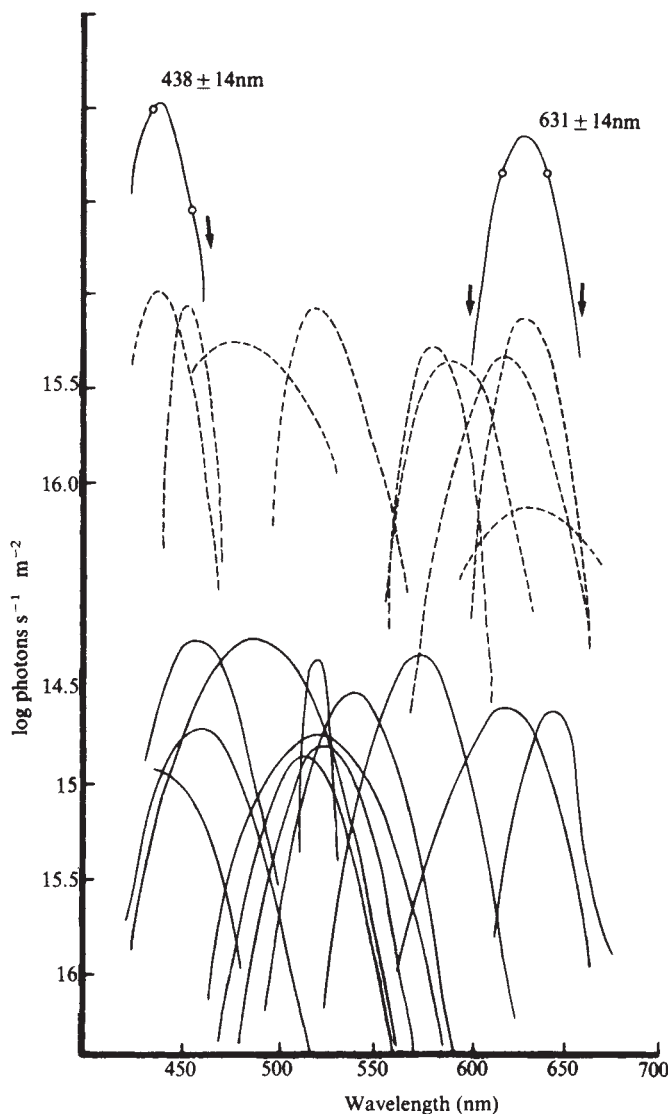


Fig. 1 Representative action spectra (wavelength selectivities) for some narrow-band cells of the fourth visual areas (V4) of monkey cortex. Action spectra were obtained by using neutral density filters and determining the minimum intensity at every wavelength to which there was a response. The upper two spectra show the responses obtained and the manner of drawing the curves to determine peak sensitivity and bandwidth. Arrows indicate there was no response at the highest intensities available. In the conventional way, curves were drawn through the experimental points by using a family of template curves, all of which were parabolas, and judging by eye which one gave the best fit. Action spectra in discontinuous lines are those of cells inhibited by light of the relevant wavelengths, those in continuous lines represent action spectra of cells excited by the relevant wavelengths.

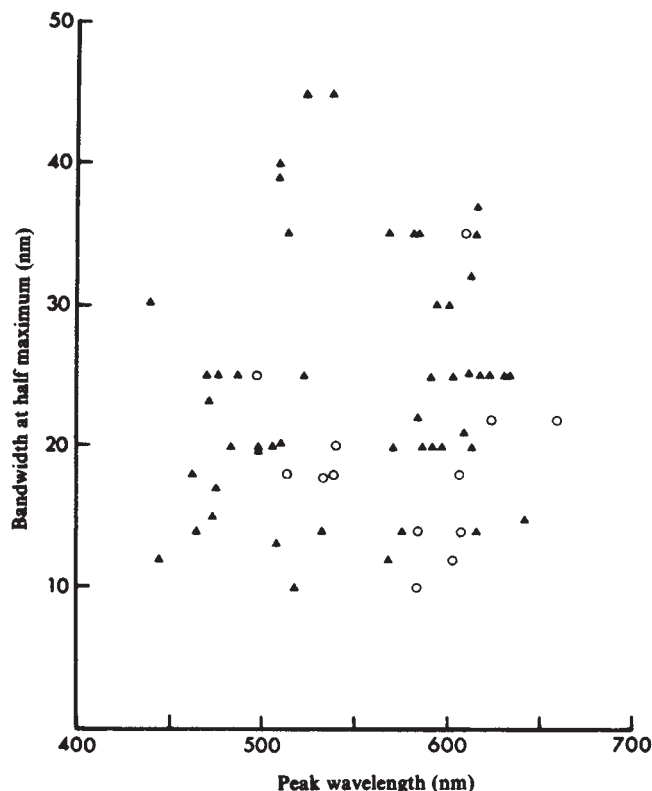


Fig. 2 A plot of peak sensitivity against bandwidth at half the maximum sensitivity for 63 action spectra obtained from 50 cells. Δ , Excitation spectra; $\circ$, inhibitory spectra.

penetrations^{16,19}. In oblique penetrations, the changes in colour preferences of successive cells, with electrode distance, are obvious and, in some, such changes are orderly. A fortunate oblique penetration, one of several, is illustrated in Fig. 4. The electrode travelled a total distance of 1,300 μm . Receptive fields of cells were large, and the shift in receptive field position was apparently random. However, the arrangement of cells with respect to one another was far from random functionally. The first three cells responded with an increase of their maintained discharge to blue, and with a decrease to red light. The next five cells were excited by green and inhibited by purple. These were followed by a cell responding to white light only. Finally, the last two cells were excited by red light and inhibited by blue. Action spectra were plotted for most of the cells. It was found that, throughout this progression, peak excitatory sensitivities were displaced from the short to the long end of the spectrum (except for the jump back from the long to the short part from cell 6 to 7). The peak inhibitory sensitivities, by contrast, were displaced in precisely the opposite direction, going through purple, the complementary, to green. Although the peak opponent sensitivities do not all cross at the white point W on the x - y chromaticity chart (Fig. 4) the order apparent when peak sensitivities are plotted on that chart is impressive and suggests that colours may be mapped in an orderly way in the cerebral cortex. The strong representation given to blue, green, purple and red is also evident in this penetration.

A study of the responses of colour-coded cells in the cortex using Land's retinex experiments

The results given above, as well as previous findings^{16,19}, attest to a high degree of order in the representation of colour in the cerebral cortex: first in generating cells with narrow wavelength selectivities and segregating them into specific cortical areas^{17,18}, then in mapping the retinal surface in these areas in a manner radically different to the retinal map in other prestriate areas¹⁴,

finally in mapping colours in an ordered way. The very segregation of cells sensitive to colour into separate cortical areas suggests that the principles governing the representation of colour in the cortex must be very different from those governing the representation of, say, form or depth. What are these principles?

Edwin Land, the originator of retinex theory, suggested to me that the responses of the cells described above were sufficiently interesting to warrant a new kind of study the purpose of which would be to ascertain the extent to which such responses correspond to, and obey, the rules of colour perception. The simple 'Mondrian' experiments in colour vision²⁵ were designed by Land to answer the classical question of why colours change so little when the wavelength-energy composition of illumination on objects is changed markedly. By using rectangles of arbitrary shape, size, surround and colour, and by choosing matte papers which reflect a constant amount of light in all directions, he created a multicoloured complex image with no recognisable objects. By illuminating this abstract scene with three bands of wavelengths whose relative energies were variable, he brought into focus the fact that colour sensations are essentially independent of energy and also independent of memory, learning, judgement, surroundings and adaptation. These perceptual experiments were so readily adaptable to electrophysiological ones that it seemed interesting to learn whether there were any cortical cells whose responses would correspond to the sensation of colour produced by the rectangles of the Mondrian display. I am much indebted to Land in the execution of the experiments described below.

Provided with this article are three filters, with cutoff points as follows: short wave, 386 and 493 nm (peak transmittance 432 nm); middle wave, 492 and 580 nm (peak transmittance 528 nm); long wave, 592 nm (peak transmittance greater than 660 nm). Each filter was chosen with a bandwidth such that an observer cannot see variegated colours through it. If one were to view a Mondrian display or a similar multicoloured scene through the red filter one would see a pattern which would be the same in disposition for all three sets of cones, even though the energy available to be absorbed by the middle-wave set and short-wave set of cones will be relatively greatly diminished. Thus all the patches which were variously coloured in unfiltered illumination will be seen as a series of light and dark areas against a reddish wash and the area which was red in unfiltered illumination will be seen as one of the lightest. Other areas such as yellow and white will also appear very light and an observer would not find it possible to predict which, among the areas that appear very light, will be red when the display is later viewed in unfiltered light. For the red area, the transition from this high lightness to a deep red in unfiltered illumination can be better understood by examining the Mondrian again, first through the green filter and after that through the blue filter. For either of these filters, as opposed to the red one, the area which is deep red in unfiltered illumination will be very dark. Thus it is as if the

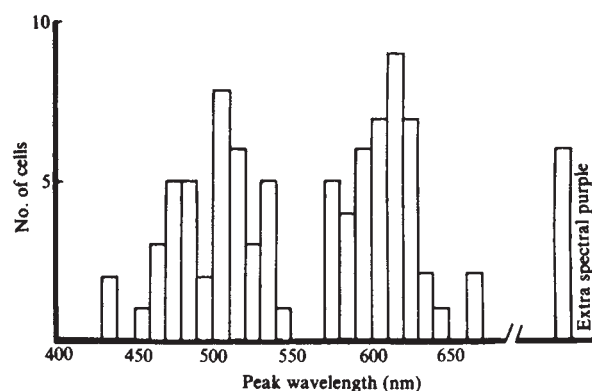
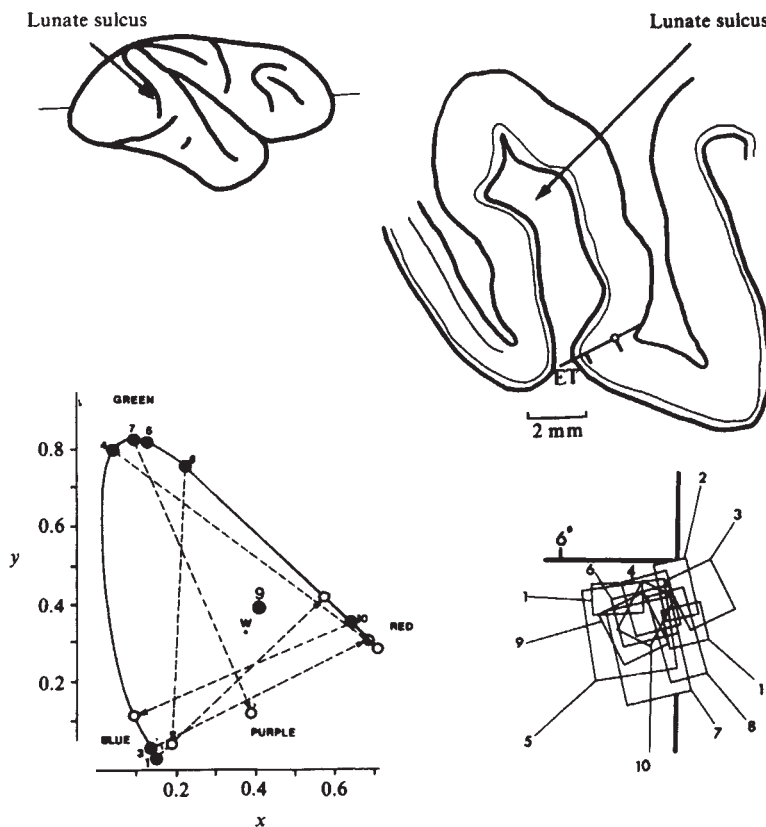


Fig. 3 Histogram of the distribution of peak sensitivities of narrow-band cells in the fourth visual areas (90 spectra obtained from 62 cells).

Fig. 4 Reconstruction of an oblique electrode penetration (ET) through the cortex of V4, made at the level indicated on the surface drawing of the brain. Receptive fields of successive cells are shown to the lower right. As in longer penetrations, shifts in receptive field position bear no obvious relation to retinal topography. The peak sensitivities of the narrow-band cells, determined from their action spectra, are entered on the x - y chromaticity chart to the left. The position of cell 9 (white) on the chart was determined from measurements of the spectral distribution of the light source and the spectral reflection coefficients of the screen. ●, Peak excitatory sensitivity; ○, peak inhibitory sensitivity. Cell 5 was lost before the inhibitory part of the action spectrum could be determined.



deep red of the area in unfiltered illumination is the consequence of the simultaneous presence of the high lightness as seen through the red filter and the two low lightnesses as seen through the green and blue filters. In general three completely independent patterns in terms of lightness and darkness will be seen through the three filters respectively. Each of these patterns as seen through one of the coloured filters will not change if a neutral filter of low, medium or rather high density is superimposed on the colour filter. It is this basic phenomenon which manifests itself as colour constancy when the relative flux in the three illuminators is altered (see below). Are there any colour-coded cells in the cortex whose response is similar to the visual experience produced by viewing Mondrian displays in variable illumination?

If to determine a red region within the field of view as being red it is necessary to illuminate the entire scene, not only by long-wave, but by middle- and short-wave light as well (and hence stimulate all three sets of cones over extended retinal regions), it follows that somewhere along the visual pathways colour-coded cells must give their optimal response when a display containing a red region, say, is illuminated not only by long- but by middle- and short-wave light as well. This was studied in the following way. Once the receptive field and the action spectrum of a cell was plotted, a multicoloured Mondrian display was placed on the screen in such a way that for a cell, say, that responded to blue only, a blue part of the Mondrian covered its receptive field. The Mondrian display was made of special matte papers, selected to have a minimum reflectance higher than 10% for any part of the visible spectrum. The display could be illuminated by three projectors, each equipped with a 750-W tungsten filament bulb and with sharp cut band-pass filters, one passing long, one middle and one short waves. The flux from each projector could be set by a rheostat. The illuminating filters were selected by Land to minimise the diversity of colour sensations from the array of coloured papers when only one projector was turned on and, while satisfying the first condition, to transmit as wide a band of wavelengths and as much light as possible²⁶.

Figure 5 shows the responses of a cell with a narrow action spectrum, responsive to red exclusively. It had no evident opponent input, either in the centre or in the surround. With the

Mondrian display in full illumination, it responded only when the red area was put in its receptive field. However, illuminating the display with long-wave light alone (equivalent to viewing it through the red filter) was totally ineffective in activating the cell. It was also unresponsive to illumination with middle- or short-wave light alone. But when all three lights were switched on simultaneously the cell gave a brisk response. This was the very condition in which the area in the cell's receptive field appeared a vivid red to human observers. Hence one's curious impression that the firing of the cell was in fact the sensation of red. In further experiments, identical responses were obtained for cells responding exclusively to green and to blue.

Table 1 Mondrian areas

Cell	White	Grey	Red	Green	Blue	Magenta	Yellow
'Red' cell	—	—	+	—	—	—	—
'White' cell	+	—	—	—	—	—	—
'Green' cell	—	ND	—	I. + II. +†	—	—	ND
'Red' cell	—	—	+*	—	I. — II. —‡	—	ND

The response of four cells to different areas on the Mondrian display when the energies coming from each area were identical. +, Presence of a response; —, no response. Unless otherwise stated each area of the display was arranged to send 69 milliwatts per steradian per square metre of long-wave light, 31 mW sr⁻¹ m⁻² of middle-wave light and 5 mW sr⁻¹ m⁻² of short-wave light. ND indicates that the area was not studied for that cell.

* There was only 21 mW sr⁻¹ m⁻² of middle-wave and 4 mW sr⁻¹ m⁻² of short-wave light at this reading.

† On a second reading for this cell, energies were arranged so that the area sent 120 mW sr⁻¹ m⁻² of long-wave, 40 mW sr⁻¹ m⁻² of middle-wave and 5 mW sr⁻¹ m⁻² of short-wave light. There was a response.

‡ On a second reading, energies were arranged so that there was 15 mW sr⁻¹ m⁻² of long-wave, 17 mW sr⁻¹ m⁻² of middle-wave and 8 mW sr⁻¹ m⁻² of short-wave light coming from that area. The cell did not respond.

While the responses of the cell illustrated in Fig. 5 are impressively clear, the same basic phenomenon was observed in many other cells, even if the responses were often not as sharp. With all such cells, however, the effects of switching off two of the three projectors, leaving only the one of the cell's preferred colour, was always the same; it led to a dramatic fall in the cell's firing rate. Figure 6 A–C shows recordings from groups of cells, all of them responsive to red alone. With the red area of the Mondrian display placed in the receptive field, switching on long wave light alone was ineffective in activating the cells of (A), gave a weak response from those of (B), and a powerful response from those of (C). In the latter, the discharge rate of the cells fell sharply after the initial outburst. Adding the middle- and short-wave lights (which, by themselves, were ineffective in activating the cells) produced the same result for all three groups—a pronounced increase in firing rate. Switching them off also produced the same result—an almost complete cessation of firing.

It is remarkable that for a cell, such as that of Fig. 5, middle- and short-wave lights should, by themselves or in combination, be totally ineffective in activating the cell and yet be instrumental in driving it in the presence of long-wave light—precisely the condition needed for a human observer to experience the sensation of a vivid red in such a multicoloured display. If these cells were themselves comparing the 'lightness records' of that area at the three wavebands, then one might have expected their

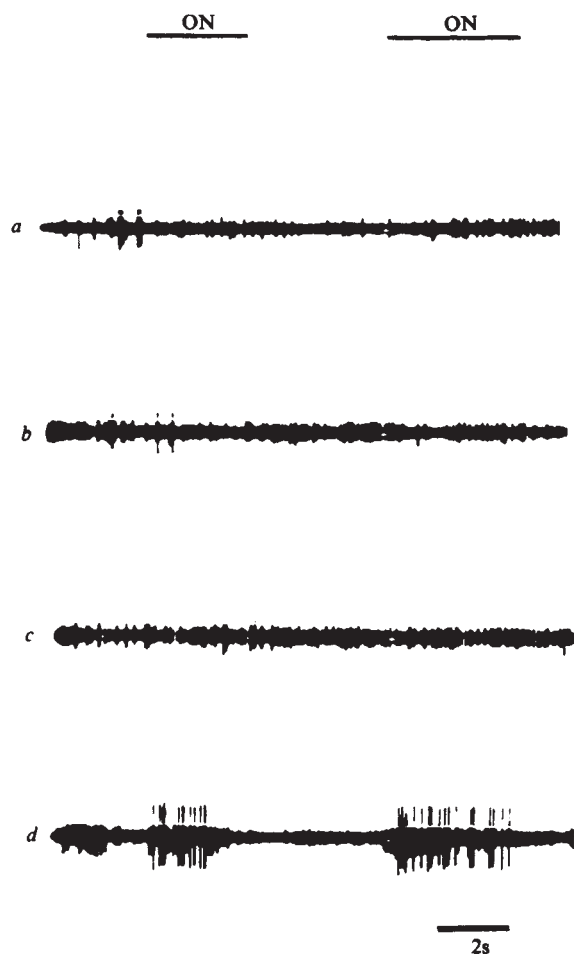


Fig. 5 The responses of a narrow-band red-orange sensitive cell (peak sensitivity 620 nm) to the red area of the Mondrian display when the display was illuminated by *a*, long-wave; *b*, middle-wave and *c*, short-wave light. In *d* the response to illuminating the display with all three projectors simultaneously is shown. To provide long-wave light, a Wratten 29 filter was placed in the light patch. The middle- and short-wave projectors had sharp cut band-pass filters with dominant wavelengths at 525 nm and 440 nm respectively.

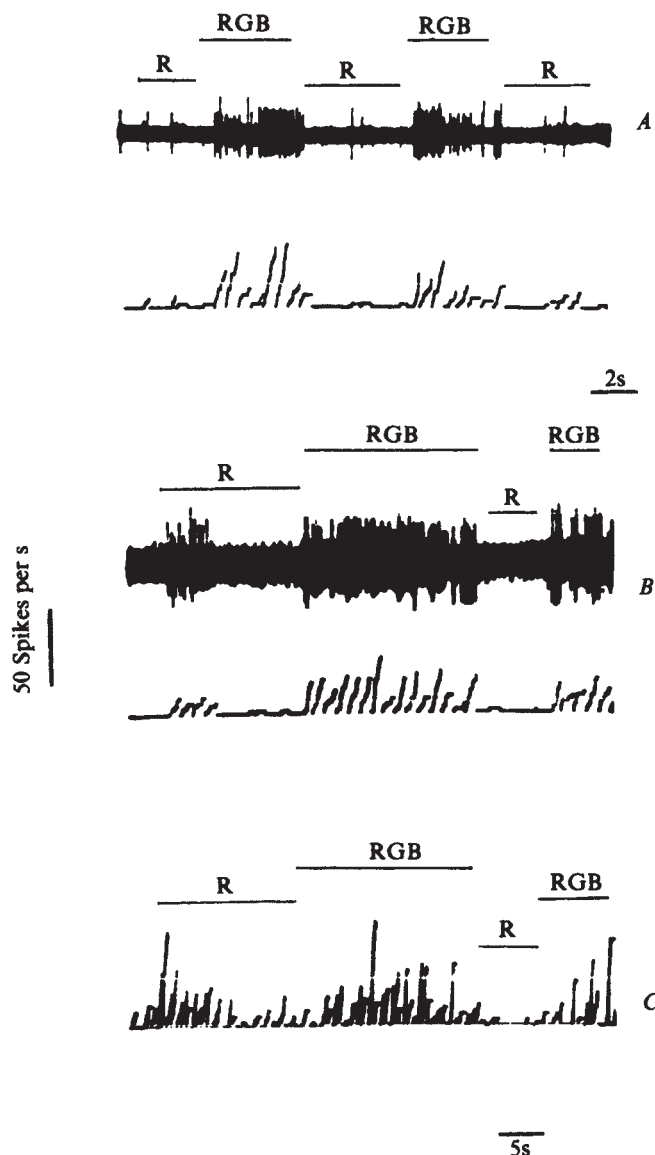


Fig. 6 The responses of three groups (A, B and C) of red-sensitive cells to the red area on the Mondrian display, when the display was illuminated with long-wave light alone (R) and when illuminated with long-, middle- and short-wave light (RGB). In the first two groups, the upper trace is the spike discharge of the cell and the lower trace the frequency of that discharge. For group C only the frequency of discharge is shown.

activity to be influenced in some way by light at each waveband. That the burst of activity occurs only when all three projectors are switched on simultaneously suggests that, if such a comparison is indeed the basis of the response, it must occur at antecedent levels. We have no clear notion whether 'lightness' at each waveband are registered by individual cells or whether more subtle interactions, requiring the activity of pools of neurones, is needed. It would therefore be premature to hazard a guess as to what level this might occur at. But V4, with the repetitive representation of each region of the retina in it^{14,16}, is as good a candidate as any. In this context, I emphasise that not all colour-coded cells in V4 behave in this 'experiential' way. Some, with action spectra restricted to the long end of the spectrum, behave much more simply, responding whenever long-wave light is switched on, no matter what part of the Mondrian display is in the receptive field. The role of these cells, and the wiring from them to the ones described above, remain to be established.

If a cell, such as that of Fig. 5, responsive exclusively to the red part of the Mondrian display when the display is trichromatically

illuminated, will nevertheless not respond to it, or do so weakly, when the same display is illuminated by long-wave (red) light alone, it becomes obvious that these responses cannot be dictated by energy-wavelength relationships. But it seemed worth repeating with these cells the experiment that Land reported on human subjects¹². Specifically, how would the cell of Fig. 5, say, respond if the amount of long-, middle- and short-wave light coming from that area on the Mondrian were made identical to the amounts of those lights coming from another part of the Mondrian, say the white, to which the cell was unresponsive. To do this, the amount of long-, middle- and short-wave lights coming off the white area were read off, one by one, using a Gamma Scientific telephotometer fitted with an equal sensitivity filter²⁶. Then, going to the other areas on the Mondrian, the amounts of all three lights were adjusted so that, from each, the identical triplet of energies reached the eye. Table 1 below shows how four narrow band cells behaved when different coloured areas on the Mondrian were made to send identical triplets of energy and put in the cells' receptive fields. It is clear that the responses of these cells could be correlated with colour alone and were independent of flux. For the green cell of Table 1, the green area of the Mondrian display was placed in the receptive field and energies adjusted so that three times more long- than middle-wave light reached the eye from it. The area still appeared green to me, a normal trichromat, and the cell gave a good response. Again, for the red cell of Table 1, the blue area was placed in its receptive field and energies adjusted so that while the area still appeared blue, it nevertheless sent twice as much long and middle, than short wave, light to the eye, and yet the cell still did not respond to it. The independence of these responses from flux is striking.

Figure 7 shows an even more remarkable response, that of a cell responding exclusively to white (of which I have encountered a few examples). When energies were made equal so that each area, when placed in the cell's receptive field, was sending an identical triplet of energies, the cell still responded only to the white area.

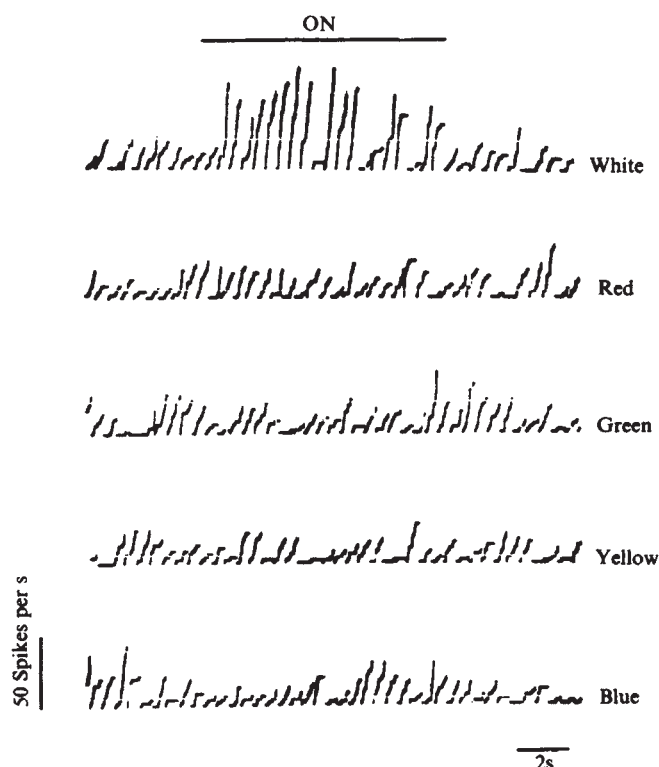


Fig. 7 The response of a cell selective to white when different areas on the Mondrian display were made to send an identical triplet of energies as that coming from the white area and placed in the cell's receptive field.

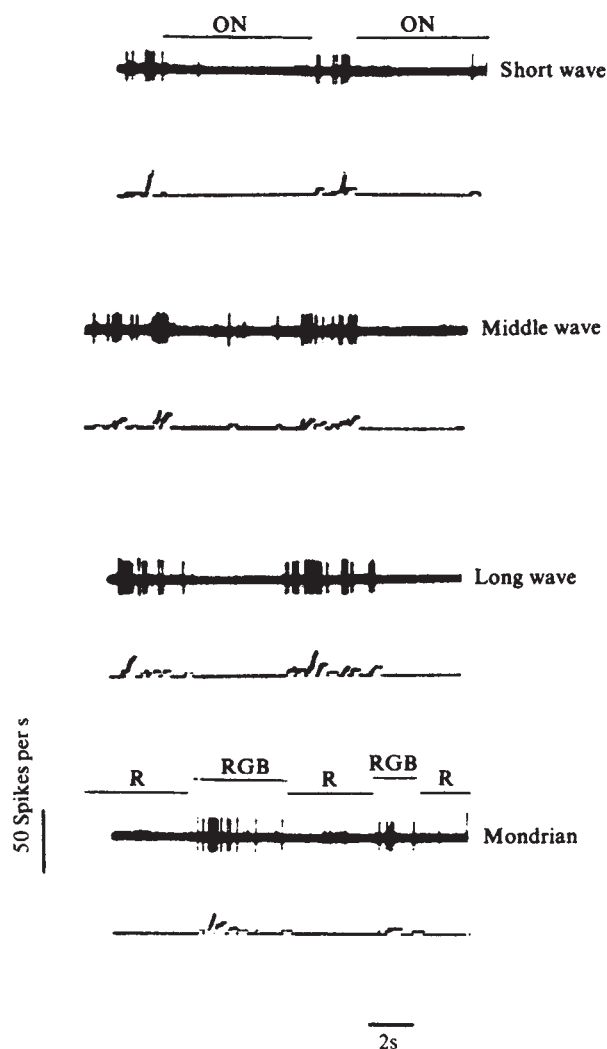


Fig. 8 Upper three traces show the response of a cell to illuminating its receptive field diffusely with short-, middle- and long-wave lights separately. The lower trace shows what happens when the red area of the Mondrian display is placed in the cell's receptive field and the entire display illuminated, first with long-wave light alone (R) and then trichromatically (RGB). The energy from the red area when the display was illuminated with long-wave light alone was $540 \text{ mW sr}^{-1} \text{ m}^{-2}$; when trichromatically illuminated it was $560 \text{ mW sr}^{-1} \text{ m}^{-2}$.

Observations such as these imply that for the single cell, just as for perception, the composition of light in terms of energy-wavelength relationships may be of little importance. Cells specifically responsive to flux, either by increasing or decreasing their firing rate, exist in the prestriate cortex (my unpublished results). However, explaining such responses in terms of energy may be wrong, as the behaviour of the cell of Fig. 8 shows. This V4 cell had a high maintained discharge which was abolished by shining short-, middle- or long-wave light diffusely onto the screen facing the animal. Switching the lights, especially the long wave one, off, led to a vigorous discharge. Superficially, then, the cell behaved as if it decreased its firing rate in response to an increase in flux. But its response using the Mondrian display contradicted this first impression. With the red area of the Mondrian display placed in its receptive field, the entire display was flooded with long-wave light (equivalent to viewing the red area of the display through the red filter). Perceptually, the red area now appeared very light, and the cell's maintained discharge was abolished, as expected. Now, adding short- and middle-wave light to the long-wave (equivalent to viewing the red area of the display¹⁵ in full illumination) and thereby increasing the energy further still led, dramatically, to an

increase in firing rate (Fig. 8). Perceptually, the effect of illuminating the Mondrian display with all three lights was to make the red area appear not only a vivid red, but also darker than it had appeared when the display was illuminated by long-wave light alone. In brief, the responses of the cell could be correlated well with human perception and were dramatically illustrated to be independent of flux.

A new neurophysiology of colour vision

The discovery of specific visual areas rich in colour-coded cells has brought us a step closer to understanding the physiology of colour vision and the nature of colour representation in the cortex. It may also provide a new approach to the study of colour vision. First, functional mapping experiments (see Fig. 4) do not reveal a topographically organised 'map' in relation to the retina. Instead they often show a representation of distinct functions (in this instance, colour) with an arrangement of the cells appropriate to that function and not necessarily following any simple topographic relation to the 'visual field'. No doubt the representation of functions also determines the pattern of anatomical connections of these areas^{13,14}. It may therefore be more meaningful to ask for these and other visual areas, how a

function is mapped, rather than how the 'visual field' is mapped.

Second, the responses of the cells reported here correspond so well with the sensation of colour that we can now, perhaps for the first time, apply identical techniques for the study of perceptual responses and those of individual cortical cells. Whereas it may be difficult to equate directly the responses of orientation selective cells with the perception of form, or of depth detecting cells with the perception of distance, there is little difficulty in equating the perception of colour with the response of the individual colour-coded cells described here. It seems timely, therefore, to apply the rules of colour perception in natural situations to the study of single cells, although we are, of course, a long way from knowing whether the cells described here are 'experiential' ones, enabling us to see the many and varied colours around us.

This work was supported by the SRC. I am indebted to Edwin Land for many helpful discussions and for his reading of this manuscript, and to Mathew Alpern for his advice in plotting the action spectra. I also thank J. Z. Young, John McCann and Luca Turin for helpful discussion, Alan Ainsworth for the electrodes, Brenda Crane for the histological assistance, and Bryan Harty for assistance during the experiments.

Received 22 November 1979; accepted 13 February 1980.

1. Young, T. *Phil. Trans. R. Soc.* **92**, 12 (1801).
2. Marks, W. B., Dobbie, W. H. & Macnichel, E. F. *Science* **143**, 1181 (1964).
3. Baker, W. B. & Rushton, W. A. H. *J. Physiol., Lond.* **176**, 56 (1965).
4. Wald, G. & Brown, P. K. *Cold Spring Harb. Symp. quant. Biol.* **30**, 345 (1965).
5. Bowmaker, J. K., Dartnall, H. J. A. & Mollon, J. D. *J. Physiol., Lond.* **298**, 131 (1980).
6. Helmholtz, H. von *Physiological Optics* Vol. II, 144 (Optical Society of America, Washington, 1924).
7. Katz, D. *The World of Colour* (Kegan Paul, Trench, Trubner, London, 1935).
8. Evans, R. M. (ed.) *An Introduction to Color* (Wiley, New York, 1948).
9. Helmholtz, H. von *Physiological Optics* Vol. II, 285-287 (Optical Society of America, Washington, 1924).
10. Hering, E. *Grundzüge der Lehre vom Lichtsinn* (Englehorn, Leipzig, 1905); quoted by Adams, G. K. *Am. J. Psychol.* **34**, 359 (1923).
11. Land, E. H. *Am. Scient.* **52**, 247 (1964).
12. Land, E. H. *Scient. Am.* **237**, 108 (1977).
13. Zeki, S. M. *Brain Res.* **14**, 271 (1969).
14. Zeki, S. M. *Brain Res.* **34**, 19 (1971).
15. Zeki, S. M. *J. Physiol., Lond.* **236**, 549 (1974).
16. Zeki, S. M. *Proc. R. Soc. B* **197**, 195 (1977).
17. Zeki, S. M. *Nature* **274**, 423 (1978).
18. Zeki, S. M. *J. Physiol., Lond.* **277**, 273 (1978).
19. Zeki, S. M. *Brain Res.* **53**, 422 (1973).
20. Hubel, D. H. & Wiesel, T. N. *J. comp. Neurol.* **158**, 275-306 (1974).
21. Wiesel, T. N. & Hubel, D. H. *J. Neurophysiol.* **29**, 1115 (1966).
22. Gouras, P. J. *J. Physiol., Lond.* **238**, 583 (1974).
23. Michael, C. R. *J. Neurophysiol.* **41**, 1250 (1978).
24. Wright, W. D. *Researches on Normal and Defective Colour Vision* (Kimpton, London, 1946).
25. Land, E. H. *Proc. R. Inst. Gr Britain* **47**, 23 (1964).
26. Land, E. H. & McCann, J. J. *J. opt. Soc. Am.* **61**, 1 (1971).

Transforming activity of DNA of chemically transformed and normal cells

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DNA fragments of chemically transformed and normal avian and murine cells induce transformation of NIH 3T3 mouse cells with low efficiencies. High molecular weight DNAs of cells transformed by DNA fragments induce transformation with high efficiencies in secondary transfection assays. It thus seems that endogenous transforming genes of uninfected cells can be activated and efficiently transmitted by transfection. These results are consistent with the hypothesis that normal cells contain genes that are capable of inducing transformation if expressed at abnormal levels.

SUBSTANTIAL evidence indicates that highly oncogenic retroviruses are recombinants between non-transforming viruses and normal cell genes. Examples of transforming viruses that have apparently originated by recombination with different cell genes include avian sarcoma viruses¹⁻³, avian myelocytomatosis virus^{4,5}, avian myeloblastosis virus⁶, avian erythroblastosis virus⁵, Moloney sarcoma virus^{6,7}, Abelson leukaemia virus⁸, Kirsten and Harvey sarcoma viruses⁹ and feline sarcoma viruses¹⁰. In the case of avian sarcoma viruses, it has been demonstrated that genetic information related to the viral transforming gene (*src*) is encoded in the genomes of many vertebrate species³. In addition, uninfected avian and mammalian cells contain a normal cell protein (p60^{src}) that is closely related to the protein encoded by the avian sarcoma virus *src* gene (p60^{src})¹¹⁻¹³. The amount of p60^{src} present in avian

sarcoma virus-transformed cells seems to be at least 100-fold higher than the amount of p60^{src} present in uninfected cells¹¹⁻¹³. These observations suggest that viral transformation may result from overproduction of normal cell proteins as a consequence of the insertion of normal cell genes into a viral genome in a manner permitting their efficient expression. If this is the case, it also seems plausible that the transforming genes of retroviruses represent only a subset of the normal cell genes that are potentially capable of inducing transformation if expressed at higher than normal levels. The present experiments indicate that DNAs of both chemically transformed and normal uninfected cells are capable of inducing transformation on transfection. The results thus provide direct support for the hypothesis that potential transforming genes are encoded in the genomes of normal avian and mammalian cells.

Transformation by sonicated DNA fragments of uninfected cells

The transforming activity of uninfected cell DNAs was assayed by transfection of NIH 3T3 mouse cells. These cells are transformable by direct integration of DNAs of avian sarcoma viruses¹⁴, avian acute leukaemia viruses¹⁵ and murine sarcoma viruses^{7,16}. In addition, NIH 3T3 cells are transformable by subgenomic fragments of murine⁷ and avian¹⁵ sarcoma virus DNAs. The efficiency of transformation by subgenomic fragments of avian sarcoma virus DNA, which contain *src* but apparently lack the normal viral transcriptional promoter, is 100–1,000-fold lower than the efficiency of transformation by intact virus DNA¹⁵. This reduced efficiency is thought to represent the probability of integration of these fragments at a site adjacent to a transcriptionally active promoter in the DNA of the recipient cells. A similar efficiency of transformation by fragments of normal cell DNAs might therefore be expected if transformation could occur by integration of normal cell genes at a site in recipient cell DNA leading to their increased expression.

The donor DNAs used in these experiments were extracted from methylcholanthrene-transformed quail cells (QT-6 cells)¹⁷, methylcholanthrene-transformed BALB 3T3 mouse cells (MC5-5 cells)¹⁸, normal chicken embryo fibroblasts and non-transformed BALB 3T3 and NIH 3T3 mouse cells. Both high molecular weight (MW) and sonicated DNAs were used in transfection assays to investigate the possibility that sonication might increase the transforming activity of uninfected cell DNAs by dissociating potential transforming genes from flanking sequences that might regulate their expression. The MWs of unsonicated DNAs were greater than 20×10^6 whereas those of sonicated DNA fragments were $0.3\text{--}3 \times 10^6$ (Fig. 1). Salmon sperm DNA (MW range $0.3\text{--}20 \times 10^6$, Fig. 1) and sonicated fragments of *Escherichia coli* DNA were included as controls to estimate the frequency of spontaneous transformation of the recipient cells.

DNAs were assayed by transfection of NIH 3T3 cells as previously described¹⁴, except that DNA-treated cells were transferred into soft agarose medium 7 days after transfection and colonies of transformed cells were counted after 2–3 weeks of further incubation. This procedure was found to reduce the background of spontaneous transformation of the recipient cells compared with that observed in assays of focus formation on the

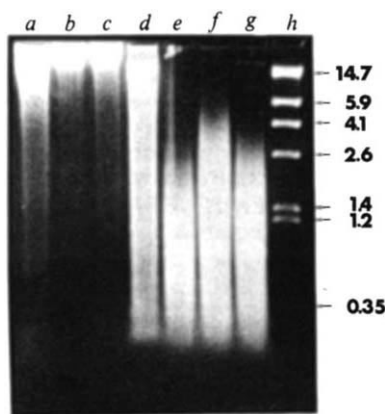


Fig. 1 Molecular weights of DNAs used in transfection assays. DNAs were extracted as described in Table 1 legend and were electrophoresed in 0.8% agarose horizontal slab gels in Tris-acetate buffer, stained with ethidium bromide and photographed under UV light¹⁴. High-MW DNAs of chicken embryo fibroblasts, NIH 3T3 cells and MC5-5 cells were electrophoresed in lanes a–c, respectively. Salmon sperm DNA was electrophoresed in lane d. DNAs of chicken embryo fibroblasts, BALB 3T3 cells and MC5-5 cells were sonicated as described in Table 1 legend and were electrophoresed in lanes e–g, respectively. The MWs of marker fragments of *Hind*III-digested λ DNA (lane h) are indicated ($\times 10^6$).

Table 1 Transformation by uninfected cell DNAs

Donor DNA	Fraction positive cultures		Transformants per μg DNA (total)
	Individual	Total	
Controls			
Salmon sperm (MW $0.3\text{--}20 \times 10^6$)	3/396	3/513	3×10^{-4}
Sonicated <i>E. coli</i> (MW $0.3\text{--}3 \times 10^6$)	0/117		
High MW ($>20 \times 10^6$)			
QT-6	1/48		
MC5-5	0/48		
CEF*	0/46	1/182	3×10^{-4}
NIH 3T3	0/40		
Sonicated fragments (MW $0.3\text{--}3 \times 10^6$)			
QT-6	5/70		
MC5-5	3/48		
CEF*	3/56	16/294	3×10^{-3}
NIH 3T3	3/80		
BALB 3T3	2/40		

High MW DNAs of avian and mammalian cells were extracted essentially as previously described²². Cells were lysed with 0.5% SDS and extracts were deproteinised by digestion with pronase, extraction with phenol and extraction with chloroform-isoamyl alcohol. DNAs were precipitated with ethanol, dissolved in SSC (0.15 M NaCl – 0.015 M sodium citrate, pH 7.0), digested with RNase A, re-digested with pronase, extracted with phenol, extracted with chloroform-isoamyl alcohol, precipitated with ethanol and dissolved in SSC. *E. coli* DNA was extracted by the same procedure after lysis of the cells by treatment with lysozyme. Salmon sperm DNA (Sigma) was dissolved in SSC, extracted with phenol, extracted with chloroform-isoamyl alcohol, precipitated with ethanol and dissolved in SSC. Fragments of DNA were prepared by sonication for 10 s using the microtip of a Branson sonifier with an output of 60 W. Recipient cultures of NIH 3T3 cells were transfected with $20\text{ }\mu\text{g}$ DNA per culture as previously described¹⁴. DNA-treated cells were transferred into semi-solid medium containing 0.25% agarose¹⁴ 7 days after exposure to DNA. Colonies of transformed cells were counted after 2–3 weeks of further incubation. Data are presented as the fraction of recipient cultures that contained transformed cell colonies (generally 1–5 colonies per positive culture). Each positive culture was considered an independent event for calculation of the transformation frequency.

* CEF DNA was extracted from normal chicken embryo fibroblasts after four to six passages in culture.

original DNA-treated plates. Control experiments indicated that the number of colonies induced by DNA of avian sarcoma virus-infected cells in this assay was similar to the number of foci induced in parallel cultures that were maintained in liquid medium ($0.1\text{--}1$ transforming units per μg DNA).

Results of transfection assays of uninfected cell DNAs are summarised in Table 1. Transfection by high MW DNAs of uninfected cells did not increase the frequency of transformation of recipient cells compared with the background of spontaneous transformation in control cultures exposed to salmon sperm or sonicated *E. coli* DNAs. In contrast, transfection by sonicated fragments of DNAs of both transformed and non-transformed cells resulted in a ~ 10 -fold increase in transformation frequency. The increased transforming activity of sonicated DNA fragments of avian and mammalian cells, compared with salmon sperm and sonicated *E. coli* DNAs, was statistically significant at the $P < 0.001$ level (χ^2 test). The frequency of transformation by sonicated DNA fragments of uninfected avian and mammalian cells was similar to the frequency of transformation by subgenomic DNA fragments of avian sarcoma virus-infected cells¹⁵.

Transformation by high molecular weight DNAs of cells transformed by DNA fragments

Individual colonies of transformed cells were picked and grown to populations of $10^8\text{--}10^9$ cells for further study. These cells had morphologies typical of transformed mouse cells and grew to

high cell densities. Cells transformed by fragments of uninfected cell DNAs did not produce retrovirus particles (assayed by sedimentable DNA polymerase activity of culture fluids¹⁹) or infectious transforming virus. In addition, transforming virus could not be rescued from these cells by superinfection with non-transforming Moloney murine leukaemia virus.

Previous studies indicated that high MW DNAs extracted from nine spontaneous transformants of NIH 3T3 cells did not induce transformation of NIH 3T3 cells in secondary transfection assays (<0.001 transforming units per μg DNA)¹⁴. In contrast, high MW DNAs of cells transformed by subgenomic fragments of avian sarcoma virus DNA induced transformation of NIH 3T3 cells with efficiencies of ~ 0.5 transforming units per μg DNA¹⁵. We therefore assayed the transforming activity of high MW DNAs of NIH 3T3 cells that were transformed by fragments of uninfected cell DNAs to determine whether DNAs of these cells were similarly capable of inducing transformation with high efficiencies.

High MW DNAs of NIH 3T3 cells and of spontaneously transformed NIH 3T3 cells isolated after exposure to salmon sperm DNA did not induce transformation in secondary transfection assays (Table 2). In contrast, high MW DNAs of NIH 3T3 cells transformed by sonicated fragments of DNAs of QT-6 cells, MC5-5 cells, NIH 3T3 cells and normal chicken embryo fibroblasts induced efficient transformation of recipient NIH

Table 2 Transformation by DNAs of NIH 3T3 cells transformed by fragments of uninfected cell DNAs

Donor DNA	Foci per μg DNA	Colonies per μg DNA
Salmon sperm	<0.002	<0.002
NIH 3T3	<0.002	<0.002
NIH (salmon sperm DNA)	<0.005	<0.002
NIH(QT-6 DNA)	0.09	0.3
NIH(MC5-5 DNA)cl 1	0.16	1.5
NIH(MC5-5 DNA)cl 2	0.15	0.3
NIH(MC5-5 DNA)cl 3	0.08	0.2
NIH(NIH DNA)	ND	0.1
NIH(CEF DNA)cl 1	0.03	0.2
NIH(CEF DNA)cl 2	ND	0.8
NIH(CEF DNA)cl 3	ND	1.1

Salmon sperm DNA, high MW DNA of non-transformed NIH 3T3 cells and high MW DNAs of transformed NIH 3T3 cells isolated after exposure to salmon sperm DNA [NIH (salmon sperm DNA)] and to sonicated DNAs of QT-6 cells [NIH(QT-6 DNA)], MC5-5 cells (three independent isolates) [NIH(MC5-5 DNA)cl 1,2,3], NIH 3T3 cells [NIH(NIH DNA)] and normal chicken embryo fibroblasts (three independent isolates) [NIH(CEF DNA)cl 1,2,3] in the experiments described in Table 1 were assayed by transfection of NIH 3T3 cells. Some cultures were maintained under liquid medium and foci of transformed cells were counted 14–17 days after transfection. Other recipient cultures were transferred into soft agarose medium 7 days after transfection and colonies of transformed cells were counted after 2–3 weeks of further incubation. ND, not done.

3T3 cells as assayed by either focus formation or colony formation in soft agarose (Table 2). The morphologies of representative foci of transformed cells are illustrated in Fig. 2. The efficiencies of transformation by DNAs of cells transformed by DNA fragments were ~ 100 -fold higher than the original efficiencies of transformation by sonicated DNA fragments. Thus, NIH 3T3 cells transformed by DNA fragments of uninfected avian and mammalian cells apparently contained transforming genes that were transmitted to new recipient cells by transfection of high MW DNA with relatively high efficiencies.

The transforming activity of restriction endonuclease-digested DNAs of three independent clones of NIH 3T3 cells transformed by fragments of chicken embryo fibroblast DNA was investigated in experiments presented in Table 3. The transforming activity of DNA of NIH(CEF DNA) clone 1 cells was abolished by digestion with *HindIII* but not by digestion with *EcoRI* or *BamHI*. In contrast, the transforming activities of DNAs of NIH(CEF DNA) clone 2 and clone 3 cells were abolished by digestion with *BamHI* but not by digestion with *EcoRI* or *HindIII*. These results indicated that transformation in secondary transfection assays was mediated by specific segments of donor DNA. In addition, it seemed that the transforming activity of NIH(CEF DNA) clone 1 cells differed from the transforming activities of NIH(CEF DNA) clone 2 and clone 3 cells, although no conclusion can be drawn concerning the relationship between the transforming activities of NIH(CEF DNA) clone 2 and clone 3 cells.

Endogenous transforming genes of uninfected cells

The findings that DNA fragments of uninfected normal and chemically transformed cells induce transformation with low efficiencies and that high MW DNAs of cells transformed by DNA fragments induce transformation with high efficiencies indicate that endogenous transforming genes of uninfected cells can be activated and efficiently transmitted by transfection. These observations are consistent with the hypothesis that normal cells contain genes that are capable of inducing transformation if expressed at abnormally high levels. A model which accounts for our results in terms of this hypothesis is presented in Fig. 3. The low efficiency of transformation by sonicated DNA fragments of uninfected cells could correspond to the probability of integration of endogenous transforming genes derived

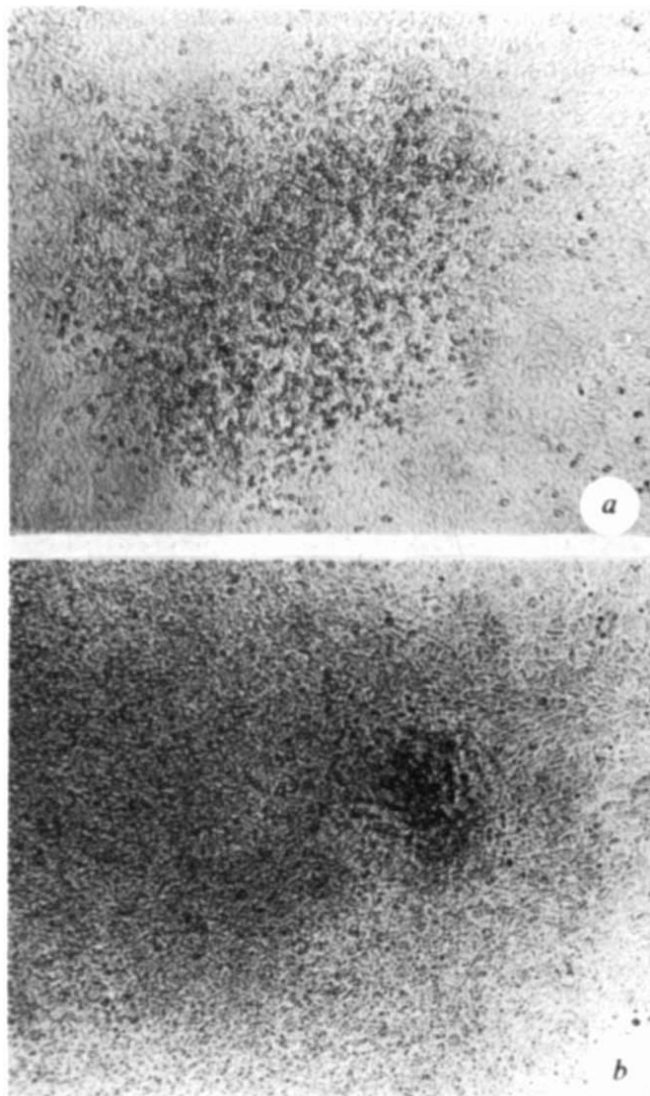


Fig. 2 Morphologies of transformed cells. Representative foci of NIH 3T3 cells transformed by DNAs of NIH(QT-6 DNA) cells (a) and of NIH(CEF DNA)cl 1 cells (b) as described in Table 2 legend were photographed with a magnification of $\times 38$.

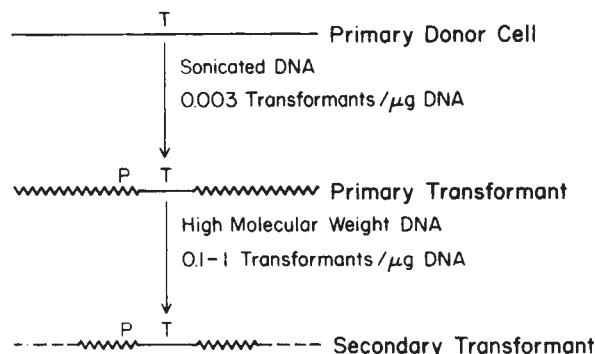


Fig. 3 Model for transfection by endogenous transforming genes. Uninfected cells used as donors of DNA contain genes that are potentially capable of inducing transformation if expressed at abnormally high levels (endogenous transforming genes). Transformation by sonicated DNAs results from integration of endogenous transforming genes (T) in the vicinity of a promoter site (P) in recipient cell DNA, resulting in abnormally high expression of the donor gene. Efficient transformation by high MW DNAs of the primary transformed cells results from transfection of secondary recipient cells by DNAs containing promoter regions (P) derived from the primary recipient genome in tandem with transforming genes (T) derived from the original donor DNA.

from the donor DNA in the vicinity of a transcriptionally active promoter in the recipient cell genome, thereby permitting abnormally high expression of the donor transforming gene. Note that the efficiency of transformation by sonicated fragments of uninfected cell DNAs (3×10^{-3} transformants per μg DNA) is similar to that by subgenomic fragments of avian sarcoma virus-infected cell DNAs ($\sim 1 \times 10^{-2}$ transformants per μg DNA)¹⁵. The transforming activity of sonicated DNA fragments, but not high MW DNAs, may indicate that the biologically active DNA fragments do not include flanking sequences that might regulate the expression of endogenous transforming genes in normal cells. The high efficiency of transformation by high MW DNAs of cells transformed by DNA fragments is consistent with transformation of the secondary recipient cells by DNAs containing promoter regions derived from the primary recipient cell genome in tandem with transforming genes derived from the original DNA fragments. Similar high efficiencies of transformation are observed in transfection assays of DNAs of cells transformed by subgenomic fragments of avian sarcoma virus DNA¹⁵ and by fragments of herpes simplex virus DNA containing the viral thymidine kinase gene²⁰. The transforming activity of avian cell DNAs in NIH 3T3 mouse cells is consistent with the transforming activity of DNAs of avian sarcoma¹⁴ and acute leukaemia¹⁵ viruses in these cells and with the biological activity of the chicken thymidine kinase gene in transfection of Ltk⁻ mouse cells²⁰.

An alternative possibility is that transformation by sonicated DNA fragments results from mutagenic activation of endo-

genous transforming genes of the recipient cells as a consequence of integration of donor DNA fragments into regulatory sequences that normally control expression of these genes. In this case also, the high efficiency of transformation by high MW DNAs of cells transformed by DNA fragments indicates that these cells contain transmissible activated endogenous transforming genes.

We are now investigating the possible relationships between the endogenous transforming genes identified here and the transforming genes of retroviruses. Molecular cloning of the transforming genes of cells transformed by fragments of uninfected cell DNAs should permit studies of these endogenous transforming genes at the molecular level and facilitate investigations of the biological activities of these genes in normal and transformed cells.

Implications for chemical carcinogenesis

The efficiency of transformation by high MW or sonicated DNAs of the two methylcholanthrene-transformed cell lines (QT-6 and MCS-5 cells) used as DNA donors in the present experiments did not differ from the efficiency of transformation by DNAs of non-transformed mouse cell lines (BALB 3T3 and NIH 3T3) or by DNAs of normal chicken embryo fibroblasts. Therefore, it seems that the two chemically transformed cell lines studied did not contain transforming genes that were efficiently transmitted by transfection of high MW DNA. These results suggest that the chemically induced transformation events that resulted in establishment of the QT-6 and MCS-5 cell lines were not transmissible by transfection. Mutational inactivation of a gene encoding a *trans*-acting regulator of the expression of an endogenous transforming gene may be an example of such a transforming lesion. Analysis of the transforming activity of DNAs of a variety of spontaneous and chemically induced neoplasms will be required to determine whether some tumours contain transforming genes that are transmissible with high efficiencies by transfection, as might result from mutational alteration of *cis*-acting regulatory sequences that control expression of endogenous transforming genes.

Since submission of this manuscript, Shih *et al.*²¹ have reported transformation of NIH 3T3 cells by high MW DNAs of 5 out of 15 chemically transformed mouse cell lines.

We thank D. M. Livingston and M. A. Lane for helpful comments on the manuscript. This research was supported by NIH grant CA 18689 from the NCI.

Received 31 December 1979; accepted 6 February 1980.

Table 3 Restriction endonuclease digestion of DNAs of transformed NIH 3T3 cells

Donor DNA	Colonies per μg DNA			
	Undigested	EcoRI	HindIII	BamHI
NIH(CEF DNA)c1	0.3	0.5	<0.03	0.5
NIH(CEF DNA)c2	0.8	0.3	0.3	<0.03
NIH(CEF DNA)c3	1.1	0.9	0.5	<0.03

DNAs of three independent clones of NIH 3T3 cells transformed by sonicated fragments of uninfected chicken embryo fibroblast DNA were digested to completion with *EcoRI*, *HindIII* and *BamHI*. The extent of digestion was determined by inclusion of λ DNA in an aliquot of each reaction mixture and analysis of the cleavage products by electrophoresis in agarose gels¹⁴. Transforming activity of undigested and digested DNAs was assayed by transfection of NIH 3T3 cells. Colonies of transformed cells in soft agarose medium were counted as described in Table 2 legend.

- Stehelin, D., Varmus, H. E., Bishop, J. M. & Vogt, P. K. *Nature* **260**, 170-173 (1976).
- Hanfusa, H., Halpern, C. C., Buchhagen, D. L. & Kawai, S. *J. exp. Med.* **146**, 1735-1747 (1977).
- Spector, D. H., Varmus, H. E. & Bishop, J. M. *Proc. natn. Acad. Sci. U.S.A.* **75**, 4102-4106 (1978).
- Sheiness, D. & Bishop, J. M. *J. Virol.* **31**, 514-521 (1979).
- Stehelin, D., Saule, S., Roussel, M., Lagrou, C. & Rommens, C. *Cold Spring Harb. Symp. quant. Biol.* **44** (in the press).
- Frankel, A. E. & Fischinger, P. J. *Proc. natn. Acad. Sci. U.S.A.* **73**, 3705-3709 (1976).
- Andersson, P., Goldfarb, M. P. & Weinberg, R. A. *Cell* **16**, 63-75 (1979).
- Baltimore, D., Shields, A. Otto, G. & Goff, S. *Cold Spring Harb. Symp. quant. Biol.* **44** (in the press).
- Scolnick, E. M., Rands, E., Williams, D. & Parks, W. P. *J. Virol.* **12**, 458-463 (1973).
- Frankel, A. E., Gilbert, J. H., Prozig, K. J., Scolnick, E. M. & Aaronson, S. A. *J. Virol.* **30**, 821-827 (1979).
- Oppermann, H. D., Levinson, A., Varmus, H. E., Levintow, L. & Bishop, J. M. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1804-1808 (1979).
- Karess, R. E., Hayward, W. S. & Hanafusa, H. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3154-3158 (1979).
- Collett, M. S., Erikson, E., Purchio, A. F., Brugge, J. S. & Erikson, R. L. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3159-3163 (1979).
- Copeland, N. G., Zelenetz, A. D. & Cooper, G. M. *Cell* **17**, 993-1002 (1979).
- Cooper, G. M., Copeland, N. G., Zelenetz, A. D. & Krontiris, T. *Cold Spring Harb. Symp. quant. Biol.* **44** (in the press).
- Lowy, D. R., Rands, E. & Scolnick, E. M. *J. Virol.* **26**, 291-298 (1978).
- Moscovici, C. *et al. Cell* **11**, 95-103 (1977).
- Lieber, M. M., Livingston, D. M. & Todaro, G. J. *Science* **181**, 443-444 (1973).
- Copeland, N. G. & Cooper, G. M. *Cell* **16**, 347-356 (1979).
- Wigler, M., Pellicer, A., Silverstein, S. & Axel, R. *Cell* **14**, 725-731 (1978).
- Shih, C., Shilo, B.-Z., Goldfarb, M. P., Dannenberg, A. & Weinberg, R. A. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5714-5718 (1979).
- Cooper, G. M. & Temin, H. M. *J. Virol.* **14**, 1132-1141 (1974).

Gene transfer in intact animals

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Resistance to methotrexate was induced in bone marrow cells of mice by transformation in vitro with DNA from a drug-resistant cell line. Transformed cells were injected in vivo and haematopoietic cells expressing resistance were selected by drug treatment of recipients. Transformed cells had elevated levels of dihydrofolate reductase and demonstrated a proliferative advantage over untransformed cells, indicating successful gene transfer.

THE classic studies of Avery, Macleod and McCarty¹ described alterations of the characteristics of one strain of *Pneumococcus* by the introduction of DNA from a second strain. This formed the basis for the identification of DNA as the primary biological material for storage of genetic information, and demonstrated the feasibility of introducing new genetic information into prokaryotic organisms. Subsequent studies examined the possibility of introducing DNA into mammalian cells. This process of gene insertion is known as transformation. There has been success in a variety of tissue culture systems using a multiplicity of techniques for inserting DNA, for example, viral vectors², cell-cell fusion, fusion to cells of a limited number of chromosomes enveloped in nuclear membranes^{3,4}, and cellular endocytosis of microprecipitates of calcium-DNA complex⁴⁻⁷. Fusion techniques give a relatively high efficiency of information insertion, whereas the calcium precipitation method allows for great selectivity in the introduction of a particular DNA species.

Many mammalian tissue culture cells will take up new DNA. However, the new genetic information must confer some advantage to the cell to demonstrate that they have transformed for a particular characteristic. For example, cell lines lacking thymidine kinase (tk^-) are readily transformed by appropriate DNA to a tk^+ status when grown in the presence of a folic acid inhibitor and thymidine^{6,7}. The presumed mechanism is the integration and functional expression of DNA specifying thymidine kinase by perhaps one cell in 10^6 . This tk^+ cell has a proliferative advantage over tk^- cells when *de novo* DNA synthesis is blocked and the cells are dependent on exogenous thymidine for DNA synthesis and proliferation.

The concept of transferring new genetic information and selecting for its expression using drug-resistance genes and drug

treatment can in principle be applied to intact animals. Certain limitations to such genetic manipulation would seem obvious. The drug used to apply selective pressure must have an acceptable level of toxicity. It must affect the particular tissue being transformed to drug resistance. Finally, if adult animals are used, only tissues demonstrating persistent proliferation throughout life would be amenable to drug-selective pressures. Anti-metabolite drugs used in cancer chemotherapy and blood-forming tissues satisfy these requirements and seem to be ideally suited for such genetic manipulations.

We describe here insertion and selection for genes conferring resistance to methotrexate in bone marrow cells of intact mice. Methotrexate (MTX) is an anti-cancer drug that acts as a folic acid antagonist by inhibiting the enzyme dihydrofolate reductase (DHFR)⁸. Mouse DNA enriched for sequences coding for dihydrofolate reductase was used to transform bone marrow cells *in vitro* and MTX was used as the selective agent *in vivo*. A well studied chromosomal marker was used to distinguish cells transformed to drug resistance from the normal cells of the recipient or from mock-transformed cells introduced as a control.

Strategy of selection of transformed cells

Mouse bone marrow cells with a distinctive chromosomal marker (T6T6)⁹ were obtained from intact animals and treated *in vitro* with a calcium microprecipitate of DNA^{6,7} from a mouse cell line selected for high resistance to MTX and containing reiterated sequences of genes coding for DHFR^{10,11}. The treated marrow, presumed to contain a few stem cells transformed to MTX resistance, was mixed in equal proportions with

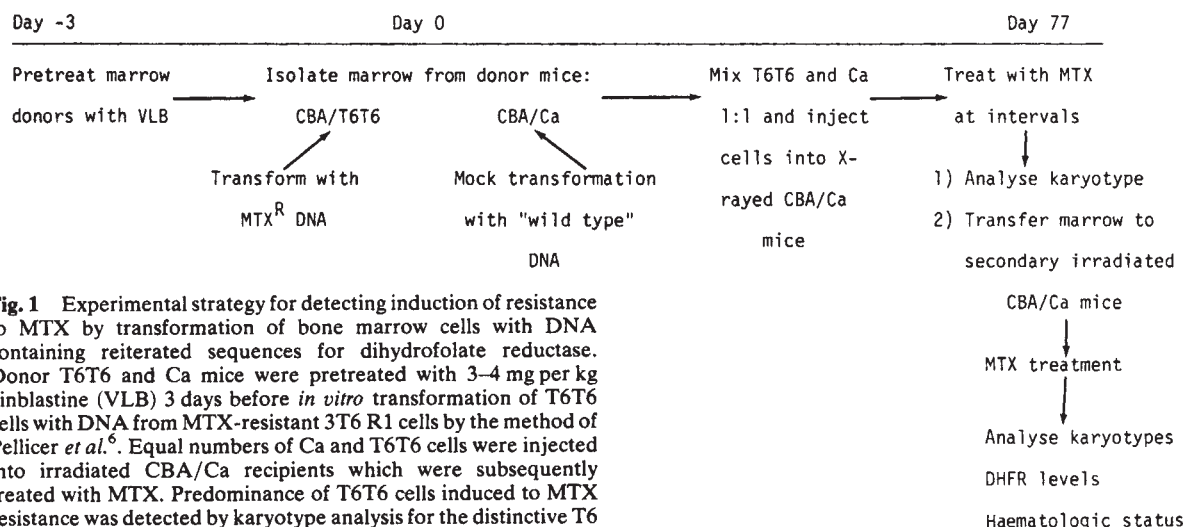


Fig. 1 Experimental strategy for detecting induction of resistance to MTX by transformation of bone marrow cells with DNA containing reiterated sequences for dihydrofolate reductase. Donor T6T6 and Ca mice were pretreated with 3–4 mg per kg vinblastine (VLB) 3 days before *in vitro* transformation of T6T6 cells with DNA from MTX-resistant 3T6 R1 cells by the method of Pellicer *et al.*⁶. Equal numbers of Ca and T6T6 cells were injected into irradiated CBA/Ca recipients which were subsequently treated with MTX. Predominance of T6T6 cells induced to MTX resistance was detected by karyotype analysis for the distinctive T6 marker chromosomes⁹.

Table 1 Karyotype analysis of marrow cells of irradiated CBA/Ca mice receiving a 1:1 mixture of control Ca and transformed T6T6 marrow cells

Recipient*	Duration of MTX treatment (days)	Karyotype (% T6T6)
Primary 1	0-24	57
Primary 2	0-32	59
Secondary 2	32-46	79
Primary 3	0-39	67
Secondary 3a	39-53	97
Secondary 3b	39-67	93
Secondary 3c	39-73	84

*Irradiated CBA/Ca recipients of the 1:1 mixture of Ca cells transformed with wild-type DNA and T6T6 cells transformed with 3T6 R1 DNA were designated 'primary' and each mouse was given a unique number. The day of infusion is designated '0'. Recipients of marrow from 'primary' animals are designated 'secondary' and bear the same identifying number. Karyotype analyses of recipient bone marrow cells were performed after the designated interval of MTX treatment. Between 50 and 100 chromosome spreads were analysed.

Table 2 Karyotype analysis of marrow cells of CBA/Ca mice receiving a 1:1 mixture of control Ca and transformed T6 marrow cells

Recipient*	Period with MTX (days)	Period without MTX (days)	Karyotype (% T6)
Primary 1	0-33	—	79
Primary 2	0-40	—	75
Primary 3	0-47	—	74
Primary 3	0-47	48-68	83
Secondary 3	47-61	—	88, 88, 100†
Primary 4	0-54	—	75
Secondary 4	54-72	—	83
Primary 5	0-65	—	96
Primary 5	0-65	66-113	63

*As in Table 1.

†Three secondary recipients.

syngeneic marrow cells lacking the chromosomal marker (Ca). These cells had been incubated in transforming conditions with control DNA lacking MTX-resistance sequences. The cell mixture was injected into genetically compatible animals that had been irradiated to reduce endogenous haematopoiesis. The infused cells gradually restored blood cell formation in these marrow-depleted recipients. During the period of reconstitution the animals were treated with MTX to produce a relative inhibition of untransformed stem cells and allow for a selective proliferative advantage for those transformed cells which had incorporated a functional gene specifying DHFR. Transformed cells were identified by their distinctive chromosomal marker¹². If there were no selective advantage, then the ratio of marked to unmarked dividing haematopoietic cells would be 50:50 or less because of the contribution of unmarked cells from the irradiated recipient. If, however, there were a proliferative advantage, then marked cells with increased levels of DHFR should be found to constitute more than 50% of the dividing marrow population.

In addition to this indirect means of detecting a population of haematopoietic cells transformed to MTX resistance, we have measured directly the levels of dihydrofolate reductase. Finally, we found that conventional measures of the haematopoietic status of MTX-treated animals provide a convenient measure of the success of the transformation procedure.

The T6 marker was selected because it is easily identifiable (low rate of false positive or negative scoring) and because of the

availability of completely syngeneic animals possessing and lacking this marker. CBA/H-T6T6 mice have the chromosomal anomaly whereas compatible CBA/Ca mice lack the marker.

Bone marrow of mice was selected for transformation by DNA because of its accessibility, high rate of proliferation, and the persistence of pluripotent stem cells throughout adult life. Of all the cell types present in the marrow, only transformation of appropriate stem cells should lead to the establishment of long-term MTX-resistant haematopoietic activity in recipient animals. It is therefore advantageous to choose cell populations rich in stem cells for transformation. Furthermore, transformation may be more efficient in dividing than in stationary cells. For these reasons we attempted to increase the level of proliferating stem cells by pretreating the donor animals with a mitotic inhibitor (vinblastine)¹³ before their use in gene transfer experiments.

MTX was chosen as the selective drug for several reasons: (1) the recent description of mouse cell lines containing highly reiterated gene sequences specifying DHFR, an enzyme conferring resistance to MTX^{10,11}; (2) the titratable suppressive effect of MTX on bone marrow cells; and (3) the potential applicability of the MTX system to human diseases¹⁴.

Haematopoietic effects of MTX treatment in the mouse

We first established a schedule of MTX treatment that would select for drug-resistant haematopoietic cells without lethality in control animals. Groups of normal CBA or C3H mice (18-25 g) were treated using a schedule of 0.5 mg per kg for four doses, 2 mg per kg for four doses and then 4 mg per kg three times weekly. This was not lethal but profoundly affected haematopoiesis. The haematocrit and tibial cellularity were found to be the easiest and most reliable haematological parameters to follow and remained depressed in animals treated continuously with MTX for at least 3 months. No difference in sensitivity to MTX was observed in the mouse strains CBA/Ca and CBA/H-T6T6.

Transformation of mouse bone marrow *in vitro*

The experimental strategy outlined in Fig. 1 was used as a guide for analysing the effectiveness of the transformation procedure.

Highly MTX-resistant mouse fibroblast Swiss 3T6 cells¹⁵ containing reiterated structural genes specifying DHFR were maintained in 4×10^{-4} M MTX and designated 3T6 R1. DNA was isolated from 3T6 R1 and from non-resistant (wild type) mouse cell lines including 3T6 (fibroblastic) and L1210 (lymphocytic leukaemia) and in later experiments from salmon sperm (Sigma). The relative ratio of DHFR synthesis and number of gene copies in 3T6 R1 and 3T6 was ~30:1 (ref. 15).

Table 3 Karyotype analysis of bone marrow and pluripotent stem cells from CBA/Ca mice receiving 1:1 mixture of control Ca and transformed T6T6 bone marrow cells

Recipient	Duration of MTX (days)	Bone marrow karyotype			
		T6T6 (%)	T6T6 (%)	Ca (%)	Mixed (%)
Primary 1	0-24	57	50	50	0
Primary 2	0-40	75	57	26	17
Primary 3	0-47	74	58	8	33

Individual spleen colonies were removed 10 days after inoculation of irradiated recipient with bone marrow cells. A single cell suspension was made from each colony and cells were incubated with colcemid $3 \mu\text{g ml}^{-1}$ for 90 min before treatment with hypotonic KCl and fixation with acetic acid/ethanol for chromosome spreads¹².

Table 4 Karyotype analysis of marrow cells of CBA/T6T6 mice receiving a 1:1 mixture of control T6 cells and transformed Ca marrow cells

Recipient	Expt	MTX treatment	Duration (days)	Karyotype (% Ca)
Primary 1	1	Yes	42	55
Primary 2	1	None	59	33
Primary 3	1	Yes	59	62
Primary 4	1	Yes	71	68
Primary 5	1	None	71	35
Primary 6	1	Yes	97	72
Primary 1	2	Yes	56	67
Primary 2	2	None	56	40

Recipient T6 mice received aliquots of a 1:1 mixture of T6 cells transformed with wild-type DNA and Ca cells transformed with 3T6 R1 DNA. Animals were either untreated or treated with MTX.

DNA co-precipitated with calcium phosphate was used to transform cells by the method of Bachetti and Graham⁴ as modified by Wigler *et al.*⁷

Equal numbers of CBA/Ca and CBA/H-T6T6 mice were injected intraperitoneally with vinblastine at 3 or 4 mg per kg 3 days before marrow was removed for *in vitro* transformation. The resulting mitotic inhibition is followed by a burst of proliferation¹³. Assays of spleen colony-forming cells (CFU-S) from animals thus treated were relatively depleted of mature cells and enriched ~threefold in pluripotent CFU-S. On the day of transformation (day 0, Fig. 1) single cell suspensions in McCoy's 5A medium with 15% fetal calf serum (FCS) were obtained from femurs and tibias. Cells from Ca and T6T6 animals were placed in separate pools. Cell suspensions of 5×10^6 in 10 ml complete medium (CM) were incubated with 1.0 ml Ca-precipitated DNA containing 40 μ g DNA as described by Wigler *et al.*⁷ for 4 h at 37 °C in 5% CO₂ in tissue culture flasks. T6T6 cells were incubated with 2 or 4 μ g DNA from 3T6 RI MTX-resistant cells, and CBA/Ca marrow cells were incubated with control DNA preparations from MTX-sensitive cells. Thereafter, loosely adherent cells were collected and centrifuged at 150g for 10 min and resuspended in DNA-free CM. After careful cell counts, Ca and T6T6 cells were combined in a ratio of 1:1 and between 5×10^6 and 5×10^7 of the combined cells were injected intravenously into recipient CBA/Ca mice in 0.3–0.4 ml in McCoy's medium with FCS. These recipients had received 850 rad from a cobalt source 24 h previously to eradicate endogenous haematopoiesis. This dose had low lethality but virtually eradicated endogenous CFU-S¹⁶. Between 48 and 96 h after injection, the recipient animals began treatment with the previously established MTX protocol.

Selection of drug-resistant marrow cells

The irradiated mice receiving mixtures of control Ca cells and T6T6 cells transformed with 3T6 R1 DNA were treated with MTX for 24–77 days. Marrow samples were obtained periodically and analysed for karyotype distribution, cellularity and CFU-S content and were injected into secondary irradiated CBA/Ca recipients (Tables 1, 2). Between about days 30 and 40 a clear increase in the percentage of bone marrow cells displaying the T6T6 marker was observed in primary recipient animals. Marrow from these mice was injected into irradiated secondary recipients which were then treated with MTX. They too showed an increased ratio of T6T6 to Ca karyotypes. This same pattern was seen in five independent experiments involving 19 primary recipient animals and 30 secondary recipients. Only two experiments during this same period failed to show a predominance of transformed karyotype.

When MTX treatment of animals receiving transformed marrow cells was stopped, the predominance of T6T6 karyotypes persisted for at least 3 weeks (primary recipient 3, Table

2) but gradually diminished by 8 weeks without treatment (primary recipient 5, Table 2). In later studies the stability of the transformed karyotype in the absence of drug selection was variable (data not shown).

To analyse whether the predominance of T6T6-marked cells involved pluripotent stem cells as well as other proliferating marrow cells, marrow was taken from selected primary recipient animals and 5×10^4 cells were injected into irradiated recipient CBA/Ca mice in a typical CFU-S assay¹⁶. Ten days later the secondary recipients were killed and individual spleen colonies removed for karyotype analysis. As seen in Table 3, the T6T6 karyotype predominated in the pluripotent marrow stem cell population. Mixed T6T6–Ca spleen colonies were also seen, presumably resulting from development of T6T6 colonies on a background of endogenous haematopoiesis in the Ca animals.

Effect of drug administration on cell predominance

We performed control experiments to determine whether T6T6-marked cells had any proliferative advantage or increased resistance to MTX and to analyse the contribution of endogenous haematopoietic repopulation in irradiated CBA/Ca animals. Experimental animals receiving an equal mixture of mock transformed Ca and mock transformed T6T6 and either untreated or treated with MTX for up to 2 months had a predominance of Ca karyotypes anticipated from the contributions of infused Ca cells and endogenous Ca cells (% Ca = 68 ± 12 ; mean $\pm$ 2 s.d.).

Finally, in two independent experiments we reversed the usual procedure and transformed Ca cells and used T6 cells as the controls. After injection of a 1:1 mixture of Ca and T6 into irradiated T6 animals they were treated with MTX or left untreated for 2 months. The Ca karyotype predominated only when MTX was administered (Table 4). This gave an unambiguous demonstration that drug therapy determined the predominant marrow population and that the karyotype *per se* did not influence predominance; that is, no inherently greater resistance to MTX was associated with either the CBA/Ca or the CBA/H-T6T6 strain.

Table 5 Haematological status of mice receiving transformed bone marrow

Recipients	MTX treatment (days)	CFU-S (per 5×10^4 cells)	Tibial cellularity (cells per 10^6)	Haematocrit (%)
Controls: saline-treated (15)	None	11.3 (5–15)	8.7 (7.1–10.6)	42 (38–47)
Controls: MTX-treated (15)	21–77	—	3.9 (3.3–4.9)	26 (23–39)
Primary 1	0–33	8.8 (7–9)	13.9	—
Primary 2	0–40	16 (13–19)	9.6	—
Primary 3	0–47	22 (18–25)	6.0	—
Primary 4	0–54	—	9.6	—
Secondary 4	54–72	—	6.6	40
Primary 5	0–65	—	8.2	47

Control animals received no irradiation or bone marrow cells and were injected with saline or MTX as described. Number of animals is indicated in parentheses in column Recipients. Numbers in parentheses in other columns indicate the range of values observed. Primary recipient CBA/Ca mice were irradiated (850 rad) and received a 1:1 mix of control Ca and transformed T6 cells before treatment with MTX for the periods indicated. Secondary recipients were irradiated and received marrow from primary recipients. CFU-S were assayed by injecting 5×10^4 marrow cells into irradiated CBA/Ca recipient mice and counting spleen colonies 10 days later¹⁶.

Haematological status of mice receiving transformed marrow

When it became apparent that cells transformed with MTX-resistant DNA predominated in the marrow of drug-treated recipient animals, simple tests were performed to assess the animals' haematological status. Animals receiving transformed bone marrow and MTX have higher haematocrits and tibial cellularity than control animals receiving MTX but no transforming DNA (Table 5). Furthermore, they have high levels of CFU-S similar to control animals that did not receive radiation or bone marrow cells. Similar results were seen in other independent experiments. Thus, the haematological status of mice receiving transformed bone marrow treated with 3T6 R1 DNA *in vitro* returned toward normal despite persistent treatment with MTX. Autopsies were performed on these animals at intervals up to 150 days after transformation, and sections of visceral organs were taken for microscopic study. No abnormalities were revealed and the animals were clinically well at the time of death.

DHFR assay

To assess whether drug resistance was related to higher enzyme levels in mice receiving transformed bone marrow, spleens were removed from primary, secondary or tertiary recipients of transformed marrow from four independent experiments and assayed for DHFR. These recipient mice were initially treated with MTX and treatment was terminated 5–7 days before collection of tissue for the DHFR assays. Appropriate controls from 12 syngeneic animals, either irradiated or not, and untreated or treated with MTX for periods of up to 6 weeks, were also used as spleen donors. In each case, conditions for control animals were chosen to match those of the experimental group. A radiometric assay for DHFR was performed on sonicated cell-free extracts of spleen^{17,18}. DHFR-specific activity was two- to fourfold greater in the spleen extracts of animals receiving transformed bone marrow than in controls (Table 6). These were considered to be underestimates of the elevations of DHFR in the haematopoietic cells of animals receiving transformed marrow, as the extracts of spleen also included stromal and capsular tissues and non-proliferating lymphoid cells.

Discussion

We have described studies aimed at demonstrating transformation of bone marrow cells to MTX resistance by gene transfer. Extrapolation from tissue culture studies suggests an efficiency of functional gene transfer of 10^{-5} or 10^{-6} for the thymidine kinase gene^{6,7}. Mice receiving 10^7 bone marrow cells receive approximately 10^4 – 10^5 pluripotent stem cells and perhaps several hundredfold greater numbers of committed myeloid and erythroid stem cells. The infusion of an effectively transformed haematopoietic pluripotent stem cell would therefore be expected to be a relatively rare event, occurring in about 1 mouse in 10, yet after 50 or more days of drug selection, animals routinely had elevated levels of the chromosomal marker indicative of cells transformed to drug resistance. Gene insertion into haematopoietic cells may therefore be easier than in some other established cell lines.

As the DHFR genes of our MTX-resistant mouse cell line seem to be similar to those of native mouse DNA, it was not possible to demonstrate unambiguously the introduction of new genes into marrow cells by the Southern blot hybridisation technique¹⁹. Consequently, we undertook a parallel series of identical experiments using the herpes simplex virus thymidine kinase (HSVtk) gene as the transforming agent, rather than DNA derived from a MTX-resistant mouse cell line. With HSVtk DNA used for marrow cell transformation, it has been possible to demonstrate virus-specific gene sequences in mouse spleen cells²⁰. Consequently, several types of drug-resistance genes may be useful in transformation studies in intact animals.

Table 6 Dihydrofolate reductase activity in spleen and karyotype analysis of bone marrow in CBA/Ca recipients receiving 1:1 mixture of control Ca and transformed T6T6 bone marrow cells

Recipient	Duration of MTX (days)	Bone marrow karyotype predominance (%)	DHFR specific activity (% control)
Primary	0–42	49	168
Primary	0–59	62*	375
Secondary	39–67	93	391
Secondary	54–72	83	183
Secondary	65–107	—	208
Tertiary	53–80	—	224

Spleen fragments were sonicated for 30 s in the cold and assayed for DHFR by the method of Hayman *et al.*¹⁸. Results are expressed as the per cent of DHFR in spleens of simultaneously killed control mice.

*This animal received transformed Ca cells and had Ca karyotype predominance. All others received transformed T6T6 cells.

The transfer of genes for drug resistance to haematopoietic cells *in vitro* and their selection in intact animals *in vivo* suggest a variety of clinical applications, for example: (1) the transfer of drug-resistance genes with the objective of enabling patients with cancer to tolerate higher doses of anti-neoplastic drugs; and (2) insertion of drug-resistance genes coupled to other genes to treat human genetic diseases such as the haemoglobinopathies.

Suppression of bone marrow haematopoiesis is one of the main limitations to intensive treatment with most clinical anti-cancer drugs. If the resistance of marrow cells could be enhanced by simple procedures, then higher doses of drugs could be used to treat cancers of other tissues. This might circumvent dose-limiting marrow-suppressive toxicity and allow better control of a variety of solid tumours. These studies indicate that mice receiving marrow transformed with MTX-resistant DNA tolerate high doses of MTX for long periods of time with nearly normal haematological parameters.

The haemoglobinopathies such as sickle cell disease and thalassaemia seem to be natural targets for treatment by gene transfer techniques. Insertion of a normally regulated and structurally normal β -globin gene should in theory correct the defect in β -thalassaemia major and sickle cell disease. However, as a normal β -globin gene should not itself confer any proliferative advantage to transformed marrow cells, it would be necessary to link it in some way to a gene whose expression is susceptible to selective pressures. Drug-resistance genes are natural candidates for this role.

We thank Dr V. Morhenn for MTX-resistant mouse fibroblast 3T6 cells, and Mrs Debra Morse, Ms Carol Le Fèvre and Ms Janice Lipeles for technical assistance. This work was supported by USPHS grants CA 15619, GM 18586 and HL 21831. M.J.C. is a recipient of an endowment from the Ambrose and Gladys Bowyer Foundation.

Received 24 September 1979; accepted 13 February 1980.

- Avery, O. T., Macleod, C. M. & McCarty, M. J. *exp. Med.* **79**, 137 (1944).
- Munyon, W., Kraiselburd, E., Davies, D. & Mann, J. J. *J. Virol.* **7**, 813–820 (1971).
- Fournier, R. E. K. & Ruddle, F. H. *Proc. natn. Acad. Sci. U.S.A.* **74**, 319–323 (1977).
- Bacchetti, S. & Graham, F. I. *Proc. natn. Acad. Sci. U.S.A.* **74**, 1590–1594 (1977).
- Maitland, N. J. & McDougall, J. K. *Cell* **11**, 233–241 (1977).
- Pellicer, A., Wigler, M., Axel, R. & Silverstein, S. *Cell* **14**, 133–141 (1978).
- Wigler, M., Pellicer, A., Silverstein, S. & Axel, R. *Cell* **14**, 725–731 (1978).
- Osborn, M. J., Freeman, M. & Huennkens, F. M. *Proc. Soc. exp. Biol. Med.* **97**, 429 (1958).
- Ford, C. E. in *Tissue Grafting and Radiation* (eds Mickle, H. S. & Douthett, J. F.) (Academic, New York, 1966).
- Schimke, R. T., Kaufman, R. J., Alt, F. W. & Kellems, R. F. *Science* **202**, 1051–1055 (1978).
- Kaufman, R. J., Bertino, J. R. & Schimke, R. T. *J. biol. Chem.* **253**, 5852–5860 (1978).
- Metcalf, D. & Moore, M. A. S. *Haematopoietic Cells*, 44–47 (North-Holland, Amsterdam, 1971).
- Smith, W. W., Wilson, S. M. & Fred, S. S. *J. natn. Cancer Inst.* **40**, 847–854 (1968).
- Cline, M. J. & Haskell, C. M. *Cancer Chemotherapy* 3rd edn (Saunders, Philadelphia, 1979).
- Kellems, R. E., Morhenn, V. B., Pfendt, E. A., Alt, F. W. & Schimke, R. T. (in preparation).
- Till, J. E. & McCulloch, E. A. *Radiat. Res.* **14**, 213 (1961).
- Hillocoat, B. L., Sweet, V. & Bertino, J. R. *Proc. natn. Acad. Sci. U.S.A.* **58**, 1632 (1967).
- Hayman, R., McCready, R. & Van Der Weyden, M. *Analyt. Biochem.* **87**, 460 (1978).
- Southern, D. M. *J. molec. Biol.* **98**, 503 (1975).
- Mercola, K. E., Stang, H., Browne, J., Salser, W. & Cline, M. J. *Science* (submitted).

Unequal crossing over in the ribosomal DNA of *Saccharomyces cerevisiae*

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Unequal sister chromatid exchanges occur at the ribosomal DNA locus of yeast during mitotic growth. The frequency of unequal crossing over, as measured by the deletion or duplication of an inserted genetic marker (LEU2), is sufficient to maintain the sequence homogeneity of the rDNA repeat units.

THE ribosomal RNA genes of yeast, *Drosophila*, *Xenopus*, silkworm and sea urchin are arranged in clusters of tandemly arrayed repeating units¹⁻⁸. Yeast rDNA consists of approximately 140 copies of a 9-kilobase repeat unit⁹. Each repeat unit codes for a 5S RNA, and for a 35S precursor RNA which is subsequently degraded to mature 25S, 18S and 5.8S ribosomal RNAs^{1,2,10,11}. Many restriction endonuclease sites have been mapped on the rDNA repeat unit^{1,2,12} and restriction analysis has shown that the repeat units are homogeneous and tandemly arranged. Restriction fragments greater than one repeat unit in length have been cloned^{2,13}.

The histone genes of sea urchin, *Drosophila* and chicken¹⁴⁻¹⁷, as well as other highly repeated sequences and satellite DNAs^{18,19} have also been shown to occur in direct tandem arrays. Repeat units in related strains or species show considerable sequence divergence; yet within any one strain the repeat units are quite homogeneous. Variation between repeat units within one cluster has been detected in *Drosophila* rDNA^{3,4} where some repeat units contain large insertions, and in *Xenopus* where length heterogeneity is due to variation in the copy number of a small internal repeat⁵. No repeat unit heterogeneity has ever been detected in the rDNA of *Saccharomyces cerevisiae*. Thus, sequence variation that is introduced by mutation in individual repeat units must be fixed throughout the entire cluster of repeats. Several mechanisms have been proposed for this process of horizontal evolution or rectification: master slave correction²⁰, democratic gene conversion²¹, saltatory replication²², and unequal crossing over^{23,24}. However, no direct experimental test of these mechanisms has been reported. This is partly due to the difficulty of classical genetic analysis with repeated genes.

We have recently reported the construction of yeast strains with insertions at the rDNA locus²⁵. A plasmid containing yeast rDNA and the *LEU2* gene was made *in vitro* and introduced into yeast by transformation (see Fig. 1). Plasmid integration occurs by homologous recombination; the large amount of homologous rDNA results in preferential integration of the plasmid at the rDNA locus. The transformants thus have a functional copy of the *LEU2* gene in the rDNA cluster which is easily scored in a *leu*⁻ chromosomal background. We have used this marker to detect the deletions and duplications that are generated by unequal crossing over (see Fig. 2). Unequal crossing over occurs during mitotic growth of haploid strains, and causes deletions and duplications of six to eight repeat units. The size of the duplications was measured by restriction enzyme analysis. The relatively small size of the duplications indicates that sister chromatid pairing in the rDNA region is an ordered process. Computer projections from the measured frequency of unequal crossing over show that this process is sufficient to explain the sequence homogeneity of the rDNA repeat units of yeast.

Insertion of *LEU2* and pBR322 at the rDNA locus

The construction of the hybrid plasmid pSZ20 has been described²⁵. The plasmid consists of two fragments of yeast DNA inserted into restriction sites on the *Escherichia coli* plasmid vector pBR322 (ref. 26). The 4.4 kilobase *Bgl*II-B fragment of rDNA is inserted at the *Bam*H-I site of the vector, and serves to direct plasmid integration into the rDNA locus. A 3.5-kilobase *Sal*I-*Xho*I fragment containing the intact *LEU2* gene^{25,27,28} is inserted at the *Sal*I site of the vector. This fragment complements a non-reverting double mutation in the chromosomal *leu2* gene, and is used for the selection of transformants^{29,30}.

Plasmid pSZ20 DNA transforms yeast with high efficiency and yields unstable transformants. This is apparently due to an origin of DNA replication on the rDNA fragment in pSZ20 (refs 25, 31) which allows autonomous replication⁴² of the free plasmid in yeast. The transformation frequency is high because chromosomal integration is not required for plasmid maintenance; the transformants are unstable due to random segregation of the plasmid molecules during mitosis. Stable integrated-plasmid derivatives are readily obtained by subcloning the initial transformants. The large amount of chromosomal

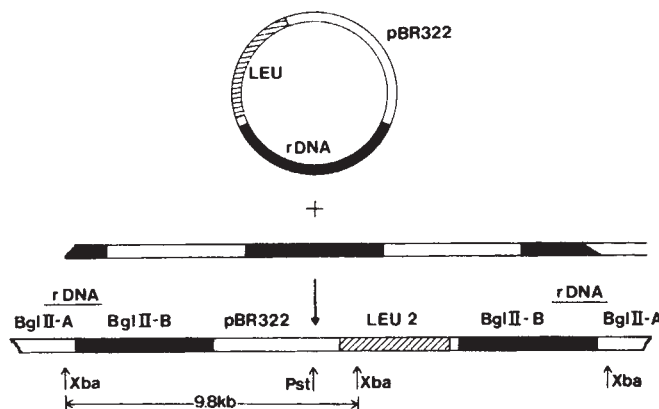


Fig. 1 Insertion of *LEU2* and pBR322 into the yeast rDNA locus. The circular plasmid pSZ20 was constructed by ligation of restriction fragments of yeast rDNA, the yeast *LEU2* gene, and the *E. coli* vector pBR322 (see ref. 25 for details of construction). The rDNA in the plasmid is a 4.4 kilobase *Bgl*II-B fragment that is approximately half of the rDNA repeat unit. Recombination with chromosomal rDNA homologous to this fragment results in plasmid integration. The integrated structure consists of *LEU2* and pBR322 sequences flanked by a direct duplication of the rDNA *Bgl*II-B fragment. Digestion of this DNA with *Xba*I generates a 9.8 kilobase fragment containing the pBR322 sequences. *Pst*I cuts near one end of the pBR322 DNA, but does not cut rDNA; thus the pBR322 remains attached to long undigested rDNA fragments unless another copy of the insertion is nearby.

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rDNA homologous to the plasmid rDNA results in a high rate of plasmid integration. The integrated-plasmid transformants are sufficiently stable through both mitosis and meiosis that the inserted marker can be genetically manipulated by standard methods.

DNA prepared from independent stable transformants was tested for the presence of restriction fragments diagnostic of the site of plasmid integration. Integration of one to four copies of the plasmid occurred at the rDNA locus in all strains tested²⁵, with multiple copies always occurring in tandem arrays. We have never observed free plasmid or plasmid integrated at other loci. Transformants containing a single copy of plasmid were most abundant (about 50%).

Restriction sites on the circular plasmid pSZ20, the chromosomal rDNA, and the integrated structure are shown in Fig. 1. The fragments generated by *Xba*I and *Pst*I are relevant to the subsequent analysis of unequal crossing over. *Xba*I generates a 9.8-kilobase fragment containing pBR322 sequences. This fragment was used for copy number measurements in which the intensity of this band was compared to the intensity of an independent single copy control. The enzyme *Pst*I cuts the plasmid once, near one end of the pBR322 DNA, without cutting rDNA at all. Therefore *Pst*I digestion of a strain with either a single or two widely separated copies of the insertion does not generate a discrete fragment carrying pBR322 DNA; the pBR322 is attached to a long undigested stretch of rDNA. Conversely, if two copies of the insertion are close together, a discrete *Pst*I fragment will be generated which spans the duplication (see Fig. 2).

Deletion of the *LEU2*-pBR322 insertion

An integrated copy of pSZ20 may be thought of as an insertion mutation. Any rectification mechanisms which operate within the rDNA cluster will tend to either eliminate or duplicate this mutation. The only functional copy of the *LEU2* gene in strain T16 is that inserted in the rDNA locus. The rate at which the insertion was spontaneously deleted was measured by a fluctuation test³² as follows. The insertion strain T16 was grown in complete medium (YPD) to stationary phase, diluted, and distributed into the wells of microtitre dishes so that about one-fourth of the wells would contain a cell. The dishes were incubated at 30 °C for 12 h to allow for six to eight generations of growth. The contents of each well were then mixed and plated onto a YPD plate. After growth at 30 °C for 2 days, the plates with colonies were replicated onto synthetic media without leucine, and scored the next day for the presence or absence of *leu*⁻ colonies.

Of 298 YPD plates, 69 (24%) had colonies. Therefore, most of the wells used to inoculate these plates started with one cell. The 67 plates scored had an average of 140 colonies per plate (9,415 total). Nine of these plates had one or more *leu*⁻ colonies, indicating that a deletion event had occurred during growth of the initial cell in the microtitre well. Each of these nine plates represents an independent deletion event. Therefore, nine events occurred during the growth of 9,415 cells, and the frequency of deletion per generation is $9/(9,415 \times 2)$, or approximately 5×10^{-4} per generation.

Deletion of the inserted *LEU2* gene was stimulated approximately 10-fold by UV irradiation to 50% survival. This observation made it much easier to pick well defined sector colonies. For this reason the analysis of unequal crossing over was done first with UV-induced sector colonies, and then repeated with spontaneously arising sectors.

Duplications generated by unequal crossing over

Unequal cross-overs can result in deletion of an insertion from one chromatid and duplication of the insertion in the other (Fig. 2). These sister chromatids are distributed to the two daughter cells after mitosis and result in a sector colony with two copies of the insertion in the *LEU*⁺ side. In contrast, a non-reciprocal

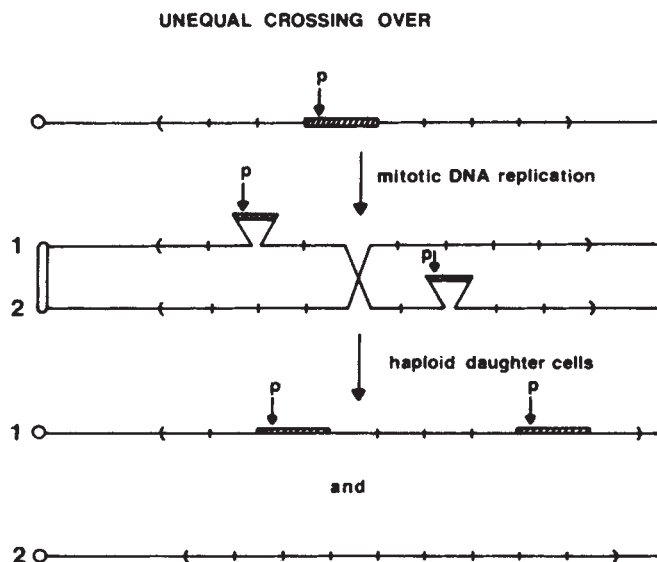


Fig. 2 Unequal crossing over can lead to duplication or deletion of the rDNA insertion. The parental strain (top) carries a single copy of the insertion, represented as a shaded box embedded in a series of tandemly arrayed rDNA repeats. Following mitotic DNA replication, the two sister chromatids pair prior to recombination. Pairing out of register leads to displacement of the two copies of the insertion relative to each other. Recombination between the displaced copies leads to one daughter cell having two copies and one having none. The daughter cells grow up side by side and form a sector colony in which cells derived from the deletion event are *leu*⁻. The event is easily detected by replica plating the colonies to media lacking leucine.

event such as looping-out excision would leave the *LEU*⁺ side of the sector with only one copy of the insertion.

The *leu*⁻ sides of 10 UV-induced sectors and 11 spontaneous sectors were tested for deletion of the insertion by colony hybridisation³⁰ with labelled pBR322 DNA. A strong hybridisation signal was obtained with the original insertion strain; none was detected with any of the *leu*⁻ strains from sector colonies or with the non-transformed parental strain (data not shown). Thus, the sector colonies result from events causing deletion of the inserted *LEU2* gene.

Duplications of the insertion in the *LEU*⁺ side of each sector were detected as follows. DNA was prepared from each strain³³ and digested with the restriction endonuclease *Xba*I to generate an identical fragment carrying the pBR322 DNA from each insertion. The DNA fragments were resolved by agarose gel electrophoresis and transferred to nitrocellulose paper by the filter blotting method³⁴. The filter was hybridised with a labelled probe containing pBR322 sequences for detection of the insertion, and *SUP3* sequences for detection of the *Xba*I *SUP3* fragment. The *SUP3* fragment serves as an independent single copy internal standard. With this method it appeared that four of the UV-induced sectors (S1, 3, 7, 9) and at least six of the spontaneous sectors (S12, 14, 15, 18, 20, 21 and perhaps S16 and 17) had a higher copy number for the insertion than the parental strain. Variation in the efficiency of DNA transfer to the filter made it difficult to obtain quantitative evidence for duplications with this method. This problem was avoided with the qualitative procedure described below.

Digestion of DNA from duplication strains with the restriction enzyme *Pst*I generates a discrete fragment carrying pBR322 sequences. In the absence of a duplication the pBR322 sequences remain attached to the rDNA, which is not digested by *Pst*I and is larger than the shear size of the isolated DNA. The detection of a discrete *Pst*I fragment is therefore strong evidence for a duplication. In addition, the size of this fragment is a measure of the size of the deletions and duplications generated by unequal crossing over. DNA from strains of all the *LEU*⁺

sectors was digested with *Pst*I; the fragments were then separated by electrophoresis through a low percentage agarose gel. In this system large fragments are well resolved and can be measured accurately. The DNA was transferred to nitrocellulose paper as above and hybridised with labelled pBR322 DNA. Hybridisation was detected with fragments of a length equal to the size of the insertion plus an integral number of rDNA repeat units (see Figs 3 and 4). Four of the ten UV-induced sectors (S1, 3, 7, 9) had duplications, ranging from 1 to 4 repeat units in length. Of the seven spontaneous sectors tested, six (S14, 15, 17, 18, 20, 21) had duplications while one did not (S19). The duplications were of 6, 7 or 8 repeat units. The data for the four UV induced and three of the spontaneous duplications are shown in Figs 3 and 4. All of the strains shown to have duplications by this method had an increased copy number of the insertion (data not shown).

The significance of the difference between UV induced and spontaneous duplications is not clear. In all cases the duplications consist of a small number of rDNA repeats (1 to 8) relative to the size of the rDNA cluster (140 repeats). This distribution could not result from a random pairing of the rDNA repeat units, and must therefore reflect an ordered pairing of the sister chromatids prior to recombination. On most of the lanes, faint bands are seen in addition to one major band. This is apparently due to secondary recombination events that result in displacement of the duplicated copies relative to each other. One of the UV-induced sectors has been completely

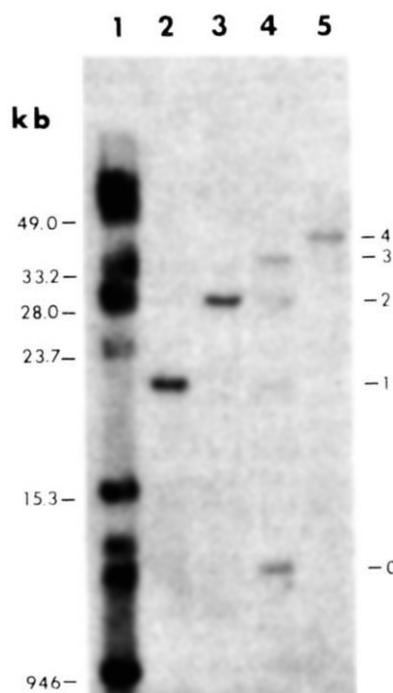


Fig. 3 Duplications produced by UV-induced unequal crossing over. DNA from the four UV-induced duplication strains was digested with *Pst*I and electrophoresed on an 0.3% agarose gel at 1 V cm^{-1} for 36 h. The molecular weight markers are a mixture of *Sa*II and *Hind*III digested fragments of λ DNA. The DNA fragments were transferred to a nitrocellulose filter and annealed with labelled pBR322 DNA. The size markers were later visualised in a separate hybridisation with labelled λ DNA. The labelled pBR322 DNA hybridised to yeast DNA fragments corresponding in size to one copy of the insertion (12.2 kilobases) plus an integral number of rDNA repeats (8.9 kilobases), as expected for a fragment spanning a duplication. Strains S1, S3 and S10 (lanes 2, 3 and 5) have fragments corresponding to duplications of 1, 2 and 4 rDNA repeat units, respectively. The pattern for S7 (lane 4) is most simply interpreted as due to randomisation by secondary recombination events, with bands that correspond to 0, 1, 2, 3 and 4 repeat unit separations of the duplicated insertion copies. In all cases faint bands are present at these positions.

randomised by such secondary events. Analysis of subclones from this culture showed that in individual cells the two insertion copies were separated by from one to five rDNA repeat units. In addition, some cells again showed randomised patterns (data not shown).

The rate of rDNA rectification

We have measured the frequency of spontaneous deletion of an insertion in the rDNA locus. These deletions are the result of unequal crossing over. Only cross-overs that occur between rDNA copies close to the *LEU2* insertion result in a detectable event. The average displacement during unequal crossing over is seven repeats out of a total cluster length of 140 repeat units, so that only 7/140 of all events are seen. The estimated frequency of deletion is 5×10^{-4} per generation; therefore, the approximate frequency of unequal crossing over within the whole rDNA cluster is 10^{-2} per generation.

Two independent calculations of the rate of rDNA rectification, based on the measured frequency and extent of unequal crossing over, are described below. We have set the initial conditions for these calculations at a 1:1 ratio of two neutral alleles. The effect of successive deletions and duplications is to cause this ratio to drift, so that the probability distribution of the allelic ratio becomes broadened. Eventually, cell lineages accumulate in which one allele or the other has become fixed in the cluster. For the purpose of these calculations, all deletions and duplications were assumed to be of seven repeat units, and to occur in alternation and randomly throughout the cluster.

Ohta³⁵ has presented a simple method for calculating the mean time required for the fixation of a neutral allele in a cluster by the reiterated action of unequal crossing over. In this model the alleles are assumed to be randomly interspersed. The number of cross-over cycles required for fixation is given by

$$t = -\frac{1}{p} [n^2 (1-p) \log_e (1-p)]$$

where p is the initial frequency of the allele in question, n is the number of cross-over units in the cluster, and one cross-over cycle is defined as one deletion event and one duplication event. We let $p = 0.5$ for an initial ratio of 1:1, and $n = 20$, for a cluster of 140 repeats with cross-over of seven repeats. The time required is 280 cross-over cycles. One cycle requires two unequal cross-overs, which occur at frequency of 10^{-2} per generation. Thus the mean time required for cross-over fixation is 5.6×10^4 generations.

We have developed a method which allows us to calculate the probability distribution of the allelic ratio in a population as a function of time (A. Blodgett and J. W. S., unpublished results). We consider the rDNA cluster to consist of 20 cross-over units, with unequal cross-overs involving deletion or duplication of one cluster of seven repeated units (on average) at a time. The probability of every allelic ratio from 20:0 to 0:20 is specified in a linear array. This array is a vector $\mathbf{p}(n)$, describing the probabilities after the n th cross-over cycle. Initially the probability of a 10:10 ratio is 1.0, and all other ratios have a probability of 0. We then calculate a square transition matrix, π , containing the probabilities of change from any one allelic ratio to any other ratio after one cross-over cycle, such that $\mathbf{p}(n+1) = \pi \times \mathbf{p}(n)$, and $\mathbf{p}(n+k) = \pi^k \times \mathbf{p}(n)$. Thus by repeated squaring of the transition matrix, an operation simple to perform on a computer, we can calculate the probability distribution vector after 1, 2, 4, 8, 16... cross-over cycles. From this probability distribution, we can calculate the probability of drift past any particular allelic ratio. The results of these calculations are summarised in Fig. 5. Drift in the allelic ratio to fixation reaches a 50% probability at 240 cross-over cycles, or about 48,000 generations. This is in good agreement with the calculation by Ohta's method. The difference is probably because complete clustering (linkage) of like alleles was assumed in this particular calculation. Drift from the original 1:1 allelic ratio to a 2:1 ratio

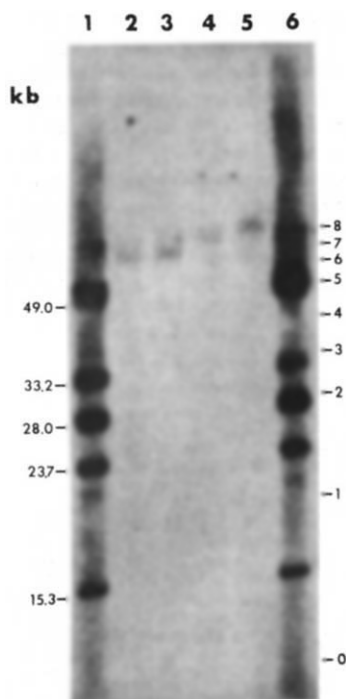


Fig. 4 Duplications produced by spontaneous unequal crossing over. The methods are as described in the legend to Fig. 3, with molecular weight markers in lanes 1 and 6. The strains used in this experiment all generate a discrete *Pst*I fragment that contains pBR322 sequences and therefore have a duplication of the insertion. The spontaneous duplication strains S21 and S14 (lanes 4 and 5) have duplications of 7 and 8 rDNA repeat units, respectively, calculated from the size of the *Pst*I fragment. Two subclones of strain S20 (lanes 2 and 3) have a duplication of 6 rDNA repeat units.

has a 50% probability at 5×10^3 generations, and a probability of 0.1 at 1,000 generations.

Discussion

We have recently shown that it is possible to integrate fragments of foreign DNA into the ribosomal RNA genes of yeast by transformation²⁵. Here we have described the use of these insertions for the analysis of unequal crossing over within the rDNA locus. Both rectification and magnification in repeated genes have been explained by unequal sister chromatid exchanges. The effect of a reciprocal unequal exchange is the generation of a deletion in one chromatid and a duplication in the other (Fig. 2). This process can lead to the elimination or fixation of a mutant allele in a cluster of repeats in the absence of selection. The process of cross-over fixation is analogous to the fixation of a neutral allele in a population by genetic drift, and is subject to a similar mathematical treatment. The variables affecting the rate of cross-over fixation by sister chromatid exchanges in a haploid are the frequency of unequal crossing over, the extent of mis-pairing (that is, the size of the deletions and duplications that are generated) and the number of repeat units in the cluster. The time required for the fixation of a neutral allele is proportional to the square of the number of cross-over units in the cluster.

The generation of deletions and duplications by unequal crossing over can also affect the copy number of repeated genes. The behaviour of the *bobbed* locus of *Drosophila*³⁶ has been studied extensively in this respect. This locus corresponds to the nucleolus organiser, the site of the tandemly repeated rDNA genes, and the *bobbed* mutation is due to a deficiency of rDNA. These deletions are unstable and revert to wild type by recombination. Tartof²⁴ has presented evidence that unequal crossing over is responsible for rDNA magnification in *Drosophila*: the

recombinational events occur in mitotic cells; gene reduction (decrease in copy number) occurs as well as copy number increase; and these events do not occur in a ring-X chromosome where the frequency of viable sister chromatid exchanges would be greatly reduced. The existence of a mechanism that is continuously generating small deletions and duplications at the rDNA locus of yeast suggests that there may be considerable variation in the rDNA copy number within a population. The average copy number in a culture is about 140 repeats per haploid genome³⁷. Natural selection alone may be sufficient to maintain the copy number near this average; alternatively some regulatory mechanism could keep the copy number closer to the optimum value.

The unequal crossing-over model of rectification and magnification does not require any new molecular mechanisms. Although sister chromatid exchanges are normally genetically invisible, they were first seen cytologically by Taylor³⁸ in his classical labelling experiments with *Vicia faba* chromosomes. Recently it has been suggested that chromosome labelling reagents such as ³H or bromodeoxyuridine cause DNA damage and induce the observed sister chromatid exchanges (SCEs). However, Yamamoto and Miklos³⁹ have detected SCEs in *Drosophila* in a ring-Y chromosome. Dicentric chromosomes resulting from sister exchanges were observed at a frequency of 0.3% in the absence of any agents which might be expected to induce this process. Unequal crossing over is familiar in *Drosophila* where it was first observed during genetic analysis of the *bar* duplication. The occurrence of unequal sister chromatid exchanges in a repeated gene cluster is therefore not surprising; in contrast, all other models of rectification require new and more complex forms of recombination.

We have measured the size of the duplications generated by unequal crossing over in yeast rDNA. In all of the events that were analysed the two copies of the insertion were relatively close to each other. The non-random distribution of the two copies has two implications. First, duplications caused by looping out of an rDNA circle including the insertion, followed by its reintegration in the sister chromatid, can be only a small fraction of the events. This mechanism would result in the two copies of the insertion being randomly distributed with respect to each other in the rDNA cluster. Second, the rDNA region of the two chromatids must pair in an ordered manner before

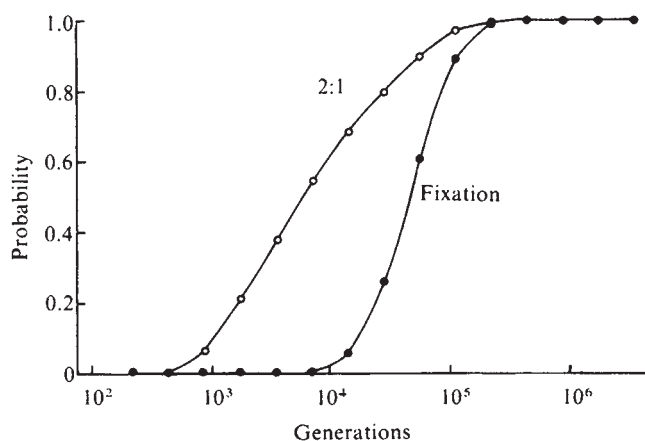


Fig. 5 Computer projections of the rectification process in yeast rDNA. The probability of drift from an initial 1:1 ratio of two alleles to a 2:1 ratio or to complete fixation was calculated as a function of time. These calculations were based on measurements of the rate and extent of unequal crossing over as described in the text. We have assumed two neutral alleles, with like alleles linked (clustered), and that deletions and duplications occur alternately, randomly throughout the cluster, and without effect on the growth rate of the cell. Drift in the allelic ratio then occurs by the random action of repeated small deletions and duplications. Drift to fixation reaches a probability of 0.5 by 4.8×10^4 generations. Drift to a 2:1 ratio should be detectable at 10^4 generations, but is unlikely to be seen at 10^3 generations.

recombination. If any rDNA repeat could pair equally well with any other, unequal crossing over would generate very large deletions and duplications that would probably be deleterious to the cell.

The frequency and extent of unequal crossing over are the two parameters required to describe the process of rectification during mitotic growth of haploid yeast. The calculations presented above and in Fig. 5 show that starting from a 1:1 ratio of two alleles in a cluster, the probability of fixation for either allele is 0.5 at 3.7×10^4 generations, and the probability of drift to a 2:1 ratio in either direction is 0.5 at 4.3×10^3 generations. The probability of drift to a 2:1 ratio after 1,000 generations is 0.1. This projection is consistent with the result of the following

Received 5 November 1979; accepted 13 February 1980.

1. Bell, G. I. *et al. J. biol. Chem.* **252**, 8118-8125 (1977).
2. Cramer, J. M., Farrelly, F. W., Barnitz, J. T. & Rownd, R. M. *Molec. gen. Genet.* **151**, 229-244 (1977).
3. Wellauer, P. K. & Dawid, I. B. *Cell* **10**, 193-212 (1977).
4. Glover, D. M. & Hogness, D. S. *Cell* **10**, 167-176 (1977).
5. Wellauer, P. K., Dawid, L. B., Brown, D. D. & Reeder, R. H. *J. molec. Biol.* **105**, 461-486 (1976).
6. Carroll, D. & Brown, D. D. *Cell* **7**, 477-486 (1976).
7. Manning, R. F., Samols, D. R. & Gage, L. P. *Gene* **4**, 153-166 (1978).
8. Wilson, F. E., Blin, N. & Stafford, D. W. *Chromosoma* **65**, 373-381 (1978).
9. Schweizer, F., MacKechne, C. & Halvorson, H. W. *J. molec. Biol.* **40**, 261-277 (1969).
10. Rubin, G. M. & Sulston, J. E. *J. molec. Biol.* **79**, 521-536 (1973).
11. Udem, S. A. & Warner, J. R. *J. molec. Biol.* **65**, 227-242 (1972).
12. Nath, K. & Bollon, A. P. *Molec. gen. Genet.* **160**, 235-245 (1978).
13. Cohen, A., Ram, D., Halvorson, H. O. & Wensink, P. C. *Gene* **3**, 135-147 (1978).
14. Schaffner, W., Gross, K., Telford, J. & Birnstiel, M. *Cell* **8**, 471-478 (1976).
15. Cohn, R. H., Lowry, J. C. & Kedes, L. H. *Cell* **9**, 147-161 (1976).
16. Lifton, R. P., Goldberg, M. L., Karp, R. W. & Hogness, D. S. *Cold Spring Harb. Symp. quant. Biol.* **17**, 1047-1052 (1977).
17. Crawford, R. V., Krieg, P., Harvey, R. P., Hewish, D. A. & Wells, J. R. E. *Nature* **279**, 132-136 (1979).
18. Botchan, M. R. *Nature* **251**, 288-292 (1974).
19. Cooke, H. J. & McKay, R. D. G. *Cell* **13**, 453-460 (1978).
20. Callan, H. G. *J. Cell Sci.* **2**, 1-8. (1967).

experiment. Petes and Botstein⁴¹ have identified a diploid strain of yeast that is heterozygous for a sequence change in the rDNA, detected as a restriction enzyme site variant. They were able to find recombinant spores heterogeneous for their rDNA. Petes (personal communication) cultured subclones of a haploid strain with a 1:1 ratio of two alleles for 1,000 generations. No change in the allelic ratio was observed. Therefore the actual frequency of unequal crossing over cannot greatly exceed the frequency measured with the rDNA insertion.

We thank Burton Beames, Albert Wu and Kathy Agne for technical assistance, and Alan Blodgett for help with the computer projections. This research was supported by a grant from the NSF.

21. Edelman, G. M. & Gally, J. A. *J. Cell Sci.* **2**, 962-972 (1970).
22. Buongiorno-Nardelli, M., Amaldi, F. & Lava-Sanchez, P. A. *Nature new Biol.* **238**, 134-137 (1972).
23. Smith, G. P. *Cold Spring Harb. Symp. quant. Biol.* **38**, 507-513. (1974).
24. Tartof, K. D. *Cold Spring Harb. Symp. quant. Biol.* **38**, 491-500. (1974).
25. Szostak, J. W. & Wu, R. *Plasmid* **2**, 536-554. (1979).
26. Bolivar, F. *et al. Gene* **2**, 95-113 (1977).
27. Ratzkin, B. & Carbon, J. *Proc. natn. Acad. Sci. U.S.A.* **74**, 487-491 (1977).
28. Hicks, J. B. & Fink, D. R. *Nature* **269**, 265-267 (1977).
29. Hinnen, A., Hicks, J. B. & Fink, G. R. *Proc. natn. Acad. Sci. U.S.A.* **75**, 1929-1933 (1978).
30. Ilgen, C., Farabaugh, P. V., Hinnen, A., Walsh, J. M. & Fink, G. R. *Genetic Engineering Vol. 1* (eds Setlow, J. K. & Hollaender, A.) 117-132 (Plenum, New York, 1979).
31. Szostak, J. W. & Wu, R. (in preparation).
32. Luria, S. E. & Delbruck, M. *Genetics* **28**, 491-504 (1973).
33. Cryer, D. R., Eccleshall, R. & Marmur, J. *Meth. Cell Biol.*
34. Southern, E. M. *J. molec. Biol.* **98**, 503-517 (1975).
35. Ohta, T. *Nature* **263**, 74-76 (1976).
36. Ritossa, F. M. *Nature new Biol.* **240**, 109-111 (1972).
37. Cramer, J. H., Bhargava, M. M. & Halvorson, H. D. *J. molec. Biol.* **71**, 11-20 (1972).
38. Taylor, J. H., Woods, P. S. & Hughes, W. L. *Proc. natn. Acad. Sci. U.S.A.* **43**, 122-127 (1957).
39. Yamamoto, M. & Miklos, G. L. G. *Chromosoma* **66**, 71-98 (1978).
40. Peterson, H. & Laughnan, J. R. *Genetics* **50**, 126-133 (1963).
41. Petes, T. D. & Botstein, D. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5091-5095 (1977).
42. Struhl, K., Stinchcomb, D. T., Scherer, S. & Davis, R. W. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1035-1039 (1979).

LETTERS

Evolution of the inclination of Phobos

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Two mutually exclusive hypotheses concerning the origin of the martian satellites have commonly been proposed: (1) they were formed in the vicinity of Mars, on orbits not very different from their present orbits, or (2) they originated far from Mars and were somehow captured¹⁻³. Hypothesis (1) had been favoured because the satellites' orbits are nearly circular and equatorial. More recent Viking results imply that the satellites are composed of carbonaceous material^{4,5}, suggesting an asteroidal origin. We show here that the satellites have remained close to their laplacian plane throughout their orbital evolution. Thus Phobos and Deimos need not have originated near the martian equator as previously believed³, but could have been captured in Mars' orbital plane instead, and later evolved to their present inclinations. This new result seems to make hypothesis (2) more plausible.

A substantial secular acceleration of Phobos has been observed^{6,7}, presumably caused by tidal dissipation in the interior of Mars. However, this mechanism seems unable to account for a drastic orbital evolution over the age of the Solar System, particularly for Deimos^{3,8}. On the other hand, Lambeck³ has shown that tides raised by Mars in the satellites, especially in Phobos, can lead to substantial changes in both the semi-major

axis and eccentricity. Because of the tidal coupling between these two elements, Phobos may once have had a very large eccentricity (≥ 0.5), permitting its semi-major axis to decrease across the synchronous orbit. Apparently Deimos has always been outside the synchronous orbit for Mars, but in the far distant past both its eccentricity and semi-major axis may also have been great. Lambeck³ concluded that Phobos, and possibly Deimos, may have been captured into an initial nearly parabolic orbit which subsequently evolved to the present orbit. However, as previously pointed out^{3,8}, tidal interactions alone cannot change the satellites' inclinations to the martian equator by more than a few degrees over the age of the Solar System. This leads to a serious objection to the capture hypothesis: if Phobos and Deimos have been affected only by tides, their initial capture orbits must have had nearly the same orientation as the martian equator, which seems improbable.

In fact the apparent small changes in the satellites' inclinations result from neglecting the precession of their orbits and the obliquity of Mars. Laplace^{9,10} has shown that a satellite orbit maintains a nearly constant inclination to its proper (laplacian) plane during a nodal precession period. This plane lies between the equatorial and orbital planes of the primary and shares a common node with them. The inclination I of the laplacian plane to the planet's equator is given by⁷:

$$mJ_2R^2a^{-3} \sin 2I = \frac{1}{2}Ma^2a_M^{-3} \sin (2\theta - 2I) \quad (1)$$

where m is the mass of Mars, J_2 its coefficient of dynamical oblateness, and R its radius; a is the semi-major axis of the satellite's orbit; M is the mass of the sun; a_M is the semi-major axis of the planet's orbit; θ is the planet's obliquity, the angle between its equatorial and orbital planes. Terms of second order in the planet's eccentricity, and of fourth order in the satellite's have been neglected in equation (1).

As the orbits of Phobos and Deimos are evolved backwards, the influence of the Sun on their motions increases while the perturbations due to the oblateness of Mars decrease. At a critical distance of about 13 martian radii¹¹, the laplacian plane switches from near the martian equator to nearer Mars' orbital plane. Because the tidal interaction does not strongly affect the inclinations of the orbits with respect to the laplacian plane, the satellites approach the martian ecliptic as they evolve back beyond the critical distance. When the satellite's eccentricity is included in the definition of the laplacian plane, the critical distance apparently increases to ~ 14 martian radii.

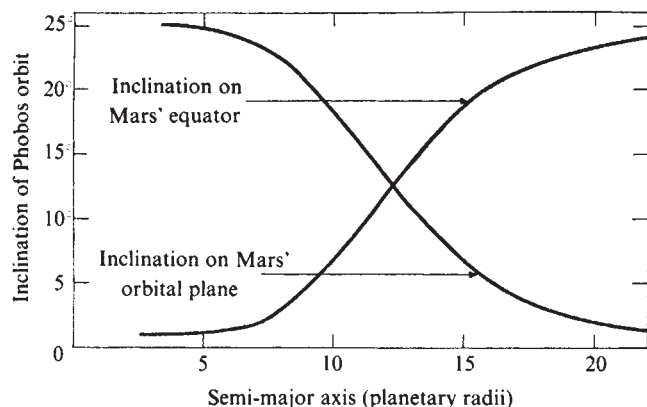


Fig. 1 Variation of the inclination of Phobos' orbit on the equatorial and orbital planes of Mars, versus its semi-major axis. For simplicity, the satellite's eccentricity has been neglected in calculating the laplacian plane.

To study the evolution of the martian satellites backwards in time, we have numerically integrated their semi-major axes, orbital eccentricities, and inclinations with respect to the laplacian plane, and simultaneously expressed their inclinations with respect to the martian equator and orbital plane. We considered both tides in the planet and tides in the satellites, using the tidal expansion of Mignard¹². This method has the capability of expressing the tidal potential relative to any reference frame; in this case, it is applied to the laplacian plane. Because Phobos and Deimos are in synchronous rotation, we have assumed that their spin axes coincide with their orbital poles throughout the orbital evolution. Thus neither tides in the satellites nor precession can alter their inclinations to the laplacian plane. The detailed method of calculation will be presented elsewhere.

The history of the semi-major axis and eccentricity depends on the frequency dependence of the tidal model. Our results are partly similar to those of Lambeck³, indicating that the eccentricity of the martian satellites may have been very high in the past. However, the orbital inclinations on the laplacian plane always remain small. Figure 1 shows the resulting variation in the inclination of Phobos' orbit to the equator and orbit planes of Mars, for a representative calculation.

As the orientation of the laplacian plane depends on the distance from Mars, we see that the orbital inclination to the equator increases beyond the critical point up to about 25°, the average obliquity of Mars¹³. At the same time, the inclination to the orbital plane of Mars falls to $\sim 1^\circ$. A similar result is expected for Deimos, but the time scale of evolution is far longer. This raises the possibility that the orbits of Phobos and Deimos crossed in the past^{3,8}.

Thus our results indicate that the martian satellites originated very close to the martian orbital plane if they ever have been outside the critical distance of Mars. In particular, this conclusion supports the capture hypothesis.

While we have replaced one 'special' plane (the martian equator) with another (the martian orbit), it is plausible that a

conceivable mechanism for capture would favour the ecliptic. In comparison, there is no obvious reason why capture should occur preferentially into the martian equator. On the other hand, the accretion hypothesis supposes that the primordial satellites' orbits lay in the laplacian plane, which depending on the distance from Mars, approaches either the equatorial or orbital plane. However, in the latter case, accretion would have occurred beyond the critical radius, which seems unlikely according to current accretionary models. Thus, while not inconsistent with an *in situ* formation, our results have removed a major difficulty with the hypothesis that Phobos and Deimos are captured asteroids.

Received 2 December 1979; accepted 15 February 1980.

- Hunten, D. H. *Icarus* **37**, 113–123 (1979).
- Pollack, J. B., Burns, J. A. & Tauber, M. E. *Icarus* **37**, 587–611 (1979).
- Lambeck, K. *J. geophys. Res.* **84**, 5651–5658 (1979).
- Veverka, J. *Vistas Astr.* **22**, 163–192 (1978).
- Duxbury, T. C. & Veverka, J. *Science* **201**, 812–814 (1978).
- Shor, V. A. *Celest. Mech.* **12**, 61–75 (1975).
- Sinclair, A. T. *Vistas Astr.* **22**, 133–140 (1978).
- Burns, J. A. *Vistas Astr.* **22**, 193–210 (1978).
- Laplace, P. *Celestial Mechanics* (Reprinted by Chelsea, New York, 1966).
- Tisserand, F. *Traité de Mécanique Céleste*, Vol II (Gauthiers-Villars, Paris, 1889).
- Goldreich, P. *Rev. Geophys.* **4**, 411–439 (1966).
- Mignard, F. *Celest. Mech.* **18**, 287–294 (1978).
- Ward, W. R. *J. geophys. Res.* **79**, 3375–3386 (1974).

Radiogenic melting of primordial comet interiors

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Comets accreted soon after the initial collapse and cooling of the solar nebula, and containing a plausible fraction of ^{26}Al , would have been significantly heated as this radionuclide decayed. Snow-and-dust balls as described by integrals of the heat conduction equation would melt in the centre if larger than 3–6 km radius. A central, low pressure vapour-droplet mixture is described here, which is conceived to be retained within an ice shell, and providing a potentially hospitable environment for elementary life forms. Refreezing after some million years produces a partially-hollow core.

Cooling and condensation of the solar nebula is thought to have occurred very rapidly, <1 Myr after nucleosynthesis of a component of the Solar System material¹. On Whipple's icy-conglomerate theory (for a recent review see ref. 2), comets also condensed at this early stage out of frozen gases mixed with solid material similar to carbonaceous chondrites, including primordial radioactive components. Whipple and Stefanik³ did investigate the probable heating due to radioactive decay, mainly by ^{40}K for they assumed a 20 Myr interval before condensation, so deriving maximum central temperatures of a few tens of Kelvins for 10-km radius icy comets. Sub-surface cometary material would thus have retained its initial pristine state—the examination of such unevolved material being a principal goal of planned cometary missions.

However, present evidence implies significant amounts of the much more effective radionuclide ^{26}Al were present in early Solar System condensates⁴, quite possibly in sufficient abundance to melt the interiors of comets⁵. This element is rather short-lived (half-life 7.4×10^5 yr) and delivered some $2,000 \text{ cal g}^{-1}$ to chondritic meteorites⁴ (assuming all the β^+ and γ -ray energy to go to heat the radio-isotope fraction to be 6×10^{-5} and the relative Al abundance of ref. 6). Comets, if accreting homogeneously out of this material⁶ with a sizeable

proportion of ices (see legend to Fig. 1), would have been subject to a smaller uniform heating rate

$$Se^{-\lambda t} \text{ where } S/\lambda = 2,000\eta \text{ cal g}^{-1} \\ \lambda^{-1} = 3.2 \times 10^{14} \text{ s}, \eta = 0.1-0.5 \quad (1)$$

This rate is $\sim 10^4$ times higher than that from ^{40}K and so on considered earlier³. To bring ice from low temperatures to melting point 90 cal g^{-1} is required and a further 80 cal g^{-1} is necessary to melt it. Thus the heat S/λ may be sufficient to melt the centre of an initial snowball comet if thermal conductivity is low and the mineral fraction is substantial, $\eta > 0.1$.

The thermal conductivity of an ice-dust comet is poorly determined because of its constitution and compactness. To secure analytical solutions I assume as previously^{3,7,8} representative constants for the density and conductivity:

solid ice + dust ($\sim 200 \text{ K}$):

$$k = 7 \times 10^{-3} \text{ cal cm}^{-1} \text{ s}^{-1} \text{ K}^{-1}, \rho = 1.0 \text{ g cm}^{-3}$$

loose snow:

$$k = 4 \times 10^{-4} \text{ cal cm}^{-1} \text{ s}^{-1} \text{ K}^{-1}, \rho = 0.25 \text{ g cm}^{-3} \quad (2)$$

with specific heat $c = 0.35 \text{ cal}^{-1} \text{ g}^{-1} \text{ K}^{-1}$, appropriate to 200 K ice. While for ice c varies strongly with temperature, for the present purposes it is only significant that k for ice is much higher than for snow.

Consider first a spherical comet of loose snow and mineral grains. The temperature in the case of uniform k is given adequately by the particular integral of the diffusion equation⁹:

$$T = T_0 + \frac{S}{c\lambda} (a \sin ar/r \sin a\alpha - 1)e^{-\lambda t}, \alpha = (\rho c \frac{\lambda}{k})^{1/2} \quad (3a)$$

$$= T_0 + \frac{S\rho}{6k} \left\{ a^2 - r^2 + a^2 O(\alpha^2 a^2) \right\} e^{-\lambda t} \quad (3b)$$

T_0 is the surface temperature, somewhat higher than the local radiation temperature T_v , which itself is just a few Kelvins far from the Sun in the 'Oort cloud' of comets, but could be some tens of Kelvins if the comets condensed early in the cooling phase of the supernova and solar nebula gases¹. The maximum, steady state value of T_0 given by radiation balance is

$$T_{0,\max} = \{T_v^4 + \rho a S / 3\sigma(1-A)\}^{1/4} \quad (4)$$

which for 10-km snowball with $\eta = 0.4$, albedo $A = 0.2$ and negligible T_v gives $T_{0,\max} \approx 37 \text{ K}$. Transient terms, dependent on unknown initial conditions, have been omitted from equation (3), as this is applicable for times greater than $\rho c a^2 / \pi^2 k \approx 7 \times 10^3 \text{ yr} \times (a/1 \text{ km})^2$ and for the source decaying little over a diffusion time $[a \approx \pi/2]$. The scale distance is $\alpha^{-1} = 4 \text{ km}$, so taking the lowest terms in the expansion (3b) is not too accurate for kilometric comets. The centre of this loose snowball comet could melt (where the temperature raised by $\Delta T = 273 \text{ K} - 37 \text{ K}(\eta a/4 \text{ km})^{1/4}$ that is if

$$\frac{\alpha a}{\sin a\alpha} > 1 + \frac{c\Delta T}{S/\lambda}, \text{ implying } a > 3-6 \text{ km} \quad (5)$$

comparable with that suggested previously⁵.

Could the pressure be high enough— $>6 \text{ mbar}$ of the triple point—to allow liquid H_2O ? The hydrostatic pressure due to self-gravity is marginally adequate: $\frac{2}{3}\pi\rho^2 G(a^2 - r^2) = 9(1 - r^2/a^2) \text{ mbar}$. A degree of cohesion between the snow crystals is also to be expected. For vapour diffusing outwards would recondense in the cooler outer layers. As cracks and interstices fill up with condensates, the interior pressure would rise, fissures may develop allowing some outgassing, but further condensation would reseal them. Thus, initially loose snow would be compacted and consolidated into an icy layer possess-

ing tensile strength, readily able to maintain pressures higher than the 6 mbar .

The relatively highly conductive (and perhaps convective) fluid and ice interior and loose snow exterior is most simply described by a two layer model: the solution corresponding to equation (3) for a shell of radius a surrounding a core of radius b , conductivities k_a, k_b is

$$T = \frac{S\rho}{6k_a} e^{-\lambda t} \frac{a^2 - b^2 + (b^2 - r^2)k_a/k_b}{a^2 - r^2} \quad \begin{matrix} r < b \\ b < r < a \end{matrix} \quad (6)$$

with $T(b) = 273 \text{ K}$ at radius b . For $k_a/k_b \ll 1$ (for example, $1/70$ by equation (2)), the interior temperature is nearly constant (273 K) and the shell temperature is a little less than the previous expression (3b). The latent heat L required in the melting or the sublimation of the ice also modifies the internal temperature: a term expressing the fact that the source has decayed while the latent heat is being delivered can be derived⁹. This term is smaller by the factor $6\lambda L b^2 / Sa^2 = 0.25(1 - \eta)b^2 / \eta a^2$ than the main term, and expresses a reduced radius b for the liquid core.

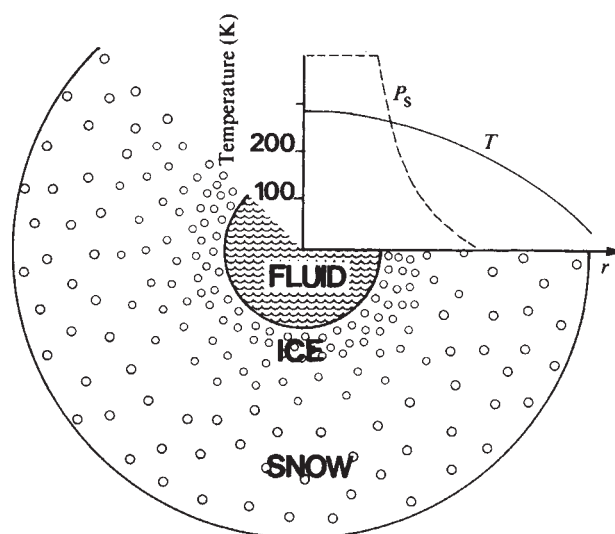


Fig. 1 A snowball comet of around 10-km radius (10^{18} g) during the initial melting phase. The temperature T and vapour pressure $p_s \sim \exp(-2,720 \text{ K}/T)$ (ref. 7) are calculated assuming convection is effective in the central vapour-droplet fluid. Comets are presumed to have accreted at a similar period to chondritic meteorites but have lower ^{26}Al radiodecay heating by a factor $\eta = 0.5-0.1$. For along with the mineral content, they retained volatiles in the form of ices of rather greater or comparable mass². The estimate for η encompasses the value $0.4-0.45$ derived if sufficient O and C to make up solar relative abundances are added to the constituents of carbonaceous chondrites⁶. The parametric values (2) are used in calculating the outer snow layer¹⁵.

The structure of a melting nucleus is certainly more complex than this two-layer model. For one thing, water is much denser (factor 4) than the snow. The gravitational pressure of a few mbar is surely too low to crack open the ice shell. Ice is a good conductor see equation (2) and in melting, readily absorbs heat from the interior fluid so the H_2O vapour pressure is probably not much higher. Could there be a central core of water surrounded by vapour? Surely not, for the gravitational potential in the vapour is flat, so the core would splash against the ice sides and fragment into drops. The central region must therefore be a mixture of one part dust and droplets, three parts vapour. The energies in droplets' dynamic motion and surface tension is of order of the potential difference: for example, typical speeds are $(\frac{2}{3}\pi\rho G h^2)^{1/2} \sim 0.2 \text{ cm s}^{-1}$ if the size h of convective cells is guessed as 10 m . The ice to snow transition is also complex. Under the vapour pressure gradient (Fig. 1) sublimation with diffusion outwards and condensation in cooler outer regions is expected,

just as in snow under sub-zero air temperatures on Earth¹⁰. This means a metamorphosis of the snow into larger ice crystals and enhanced thermal conductivity due to vapour transport:

$$k_{\text{eff}} = k + \zeta \frac{L}{RT} \partial p_s / \partial T$$

Assuming $\zeta = \varepsilon v_{\text{th}}/3$, with $\varepsilon = 1$ mm a typical hole size, the additional conductivity is found to exceed k for snow, equation (2), above 200 K. Thus in the 10-km snowball sketched in Fig. 1, a 1-km thick icy layer with enhanced conductivity is expected, and a further 2 km of metamorphosing snow.

There may be additional chemical heating which counteracts latent heat effects. If there had been little chance for radiative processing and polymerisation of the icy grains¹¹ then states of lower chemical energy would be accessible in the vapour-liquid-mineral dust interior, through catalysed chemical and perhaps biological^{8,12} processes. Supposing Solar System abundance of C and 0.2–0.5 eV per C-atom (compare with Van der Waals forces at 0.2 eV per bond; H₂CO polymerisation at 0.37 eV) the extra heat amounts to 80–200 cal⁻¹. Being released quickly from newly-melted material, it makes the effective latent heat negative and may fully counterbalance the thermal capacity (90 cal g⁻¹). If so, once melting has started, the liquid-vapour core grows very quickly (the chemical time scale) and equation (6) describing a fully-developed core is rapidly obtained ($\lambda t \ll 1$).

As the radioactivity decays, the comet centre gradually refreezes, with an exponential decay time of λ^{-1} . Convection may enhance thermal transport, but anyway the ice-water-vapour interior is relatively highly conducting, so the shell-core solution (6) with $k_a \ll k_b$ is appropriate. Allowance for the latent heat can again be made⁹, but only expresses a slowing down in refreezing. In structural terms, freezing proceeds through deposition of frost and hail on the interior of the ice shell, resulting eventually in a hollow core.

Thus a 10-km snowball comet (10⁸ g) would have developed a large fluid core with a 1-km thick icy shell, while the snow surrounding it underwent extensive processing through sublimation, diffusion and recondensation, before the core refroze (with crystallisation effecting further differentiation) in ~ 1 Myr. The hollow core resulting on refreezing provides an explanation for the splitting of some disintegrating comets into fragments of comparable size^{3,13}. Of those observed over the past 25 yr, comets Wirtanen, Humason, Schwassmann-Wachmann¹⁴ and Chiron (if it is a comet) seem big enough to have possessed large fluid cores, although comets such as Encke and Halley may have been bigger before centuries of outgassing. The central fluid core would have provided an excellently-protected environment for elementary biological systems¹², particularly for colonies of bacteria which could survive the refreezing and 4,500 Myr isolation, and be released from comets evaporating and disintegrating on approach to the Sun.

I thank Chandra Wickramasinghe and Mike Edmunds for helpful discussions. I acknowledge support under a UK SRC contract.

Received 23 November 1979; accepted 19 February 1980.

A search for ultra high-energy γ -ray bursts from celestial sources

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We report here the details of an experiment carried out at mountain altitude, at Ootacamund (2.2 km above sea level), India between March 1977 and March 1979, to detect bursts of high energy ($\sim 5 \times 10^{13}$ eV) γ rays from celestial sources. Not having detected any such bursts, we give 99% confidence level upper limits both to their flux and to the primordial mini-black hole explosion rate in the vicinity of the Solar System.

The apparatus consists of four 20-cm deep liquid scintillation detectors, each 1 m² in area located at the corners of a square of side 11 m. Each scintillator is viewed by a RCA 8575 photomultiplier with the associated electronics set at a threshold of 0.33 of the average pulse height for a vertically penetrating incident cosmic ray muon. The scintillators are, therefore, fully sensitive to single charged particles. Low-energy air showers are selected by a fourfold coincidence of the pulses from the scintillators with a resolving time of 1 μ s. The observed shower rate is 0.55 s⁻¹ with a negligible contribution from chance coincidences. The primary energy threshold is difficult to calculate precisely; although we can approximate it at $\sim 5 \times 10^{13}$ eV. We looked for events in which a rapid sequence of air showers occurred with a short time separating them. Specifically all events in which three or more showers occurred with inter-shower time separations of 10 ms or less were selected by the burst detection system discussed in detail elsewhere¹. The event times were recorded to an accuracy of 1 ms in UTC (coordinated universal time) and the inter-shower time separations to an accuracy of 1 μ s. The expected chance event rates of the '3', '4' and '5 or more' type bursts due to just poissonian fluctuations are 1.44, 7.9×10^{-3} and 4.3×10^{-5} per day respectively. Thus while the '3' and '4' type of events are trivial, a '5 or more' type of burst is very significant if recorded in an operation period of a year or two. To discriminate against spurious bursts generated by local phenomena like electrical discharges, a dummy system with electronics identical to that of the burst detection system (except that the photomultipliers were absent) was also operated during the experiment. A signal from any one of the four dummy detectors was used to flag out a possible noise event.

In an effective operation time of 4.78×10^7 s (1.51 yr), no '5 or more' type of burst was observed. The numbers of '3' and '4' type of events observed during the same period are 810 and 4 compared with the expected 797 and 4 events respectively due to chance. The absence of any '5 or more' type of bursts allows us to set an upper limit on the frequency of γ -ray bursts.

The 99% confidence level upper limit to the flux of γ -ray bursts, characterised by at least five γ -ray photons each of energy $\geq 5 \times 10^{13}$ eV arriving within a time interval of 0.1 s, is

$$\frac{4.6}{\pi(30 \text{ m})^2 \times 4.78 \times 10^7 \times 1} = 3.4 \times 10^{-11} \text{ m}^{-2} \text{ s}^{-1} \text{ sr}^{-1}$$

Here we assumed that the collection area is that of a circle with a radius of 30 m and the solid angle, 1 sr.

It is obvious that the same results can be used to set an upper limit to the number of exploding mini-black holes postulated by Hawking²⁻⁸. Using the same calculation procedure as Fegan *et al.*⁹, who carried out a similar experiment, we arrive at a 99% confidence limit of 2.7×10^3 mini-black hole explosions pc⁻³ yr⁻¹. This compares favourably with the limit of 6×10^3 pc⁻³ yr⁻¹ set by Fegan *et al.* for a threshold energy of

1. Reeves, H. *Astrophys. J.* **231**, 229 (1979).
2. Whipple, F. L. & Huebner, W. F. A. *Rev. Astr. Astrophys.* **14**, 143 (1976).
3. Whipple, F. L. & Stefanik, R. P. *Mém. Soc. R. Sci. Liège Sér. 5*, **12**, 33 (1966).
4. Lee, T., Papanastasiou, D. A. & Wasserborg, G. J. *Geophys. Res. Lett.* **3**, 109 (1976); *Astrophys. J.* **211**, L111 (1977).
5. Geiss, J. *Comments on Meteors and Meteorite Research and Comet Probing with respect to the Origin of the Solar System* (ESA Workshop on Cometary Missions, ESOC Darmstadt, 1978).
6. Wasson, J. T. *Meteorites, Classification and Properties* Ch. 7 (Springer, Berlin 1974).
7. Dobrovolskii, O. & Markovich, M. Z. *IAU Symp.* No. 45, 287 (1972).
8. Irvine, W. M., Leschine, S. B. & Schloerb, F. P. *Nature* **283**, 748 (1980).
9. Wallis, M. K. *Astrophys. and Relativity* (Preprint, University College, Cardiff, 1979).
10. Palm, E. & Tweitheid, M. J. *geophys. Res.* **84**, 745 (1979).
11. Goldanski, V. *Nature* **279**, 109 (1979).
12. Hoyle, F. & Wickramasinghe, N. C. *Lifeloud* (Dent, 1978).
13. Sekanina, Z. *Sky Telesc.* **51**, 386 (1976).
14. Roemer, E. *Mém. Soc. R. Sci. Liège Sér. 5*, **12**, 23 (1966).
15. *American Institute of Physics Handbook* 2nd edn (1963).

$\sim 10^{14}$ eV. Porter and Weekes¹⁰, using an atmospheric Cerenkov technique, obtained an upper limit for mini-black hole explosion rate of $3 \times 10^4 \text{ pc}^{-3} \text{ yr}^{-1}$ at a threshold of 5×10^{12} eV. According to Page and Hawking⁷ and Carr⁸ who discussed in detail the observable consequences of primordial mini-black holes in the so-called elementary particle model, 10–30% of the rest mass (10^{15} g) of an exploding black-hole will result in the emission of a burst of about 10^{30} photons each of energy $\sim 5 \times 10^{12}$ eV, over a time scale of 0.1 s. The above limits have to be considered in the context of this prediction.

We thank A. R. Apte, J. A. Joshi and S. Ramani for their experimental assistance.

Received 18 January; accepted 12 February 1980.

1. Bhat, P. N. *et al.* Preprint (1979).
2. Hawking, S. W. *Nature* **248**, 30 (1974).
3. Hawking, S. W. *Commun. math. Phys.* **43**, 199 (1975).
4. Wald, R. M. Preprint EFI 75-13 (University of Chicago, 1975).
5. Parker, L. *Phys. Rev. D* **12**, 1519 (1975).
6. DeWitt, B. S. *Phys. Rep.* **19C**, 295 (1975).
7. Page, D. N. & Hawking, S. W. *Astrophys. J.* **206**, 1 (1976).
8. Carr, B. J. *Astrophys. J.* **206**, 8 (1976).
9. Fegan, D. J. *et al.* *Nature* **271**, 731 (1978).
10. Porter, N. A. & Weekes, T. C. *Nature* **277**, 199 (1979).

Electrogenative reduction of nitric oxide

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Electrogenative processes are those in which favourable thermodynamics and kinetic factors are utilised in an electrochemical cell to generate by-product electricity while bringing about a desired reaction. The cell actually functions as a reactor to produce a desired chemical or occasionally to destroy an undesired one. Electrode potentials are generated by reactions at the electrodes; they are frequently different from those of conventional electrosynthesis. Here, we describe an exploratory study of the electrogenerative reduction of nitric oxide to favour ammonia production as well as the effect of electrocatalyst, external load, and other parameters on product distributions.

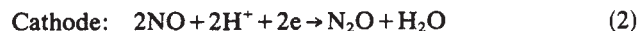
Nitric oxide can be produced in several ways. One method would be through concentration from a polluting effluent gas stream. Another possibility would be passage of an effluent stream through an electrogenerative cell removing nitric oxide while generating d.c. electricity.

The reduction of nitric oxide has been of special interest from a basic and environmental viewpoint¹, both electrochemical reductions^{2–5} and conventional catalytic hydrogenations^{6–10} having been investigated. For the 'electrogenative' reduction of nitric oxide, the free electrolyte phase is bounded by porous Teflon-backed, gas-permeable, liquid-impermeable, electrodes which are connected through an external variable resistor in series with an ammeter¹¹. Hydrogen at atmospheric pressure reacts in one electrode compartment, while nitric oxide reacts at the other with protons and electrons from the external circuit to generate current at potentials determined by reactions at each electrode. Details of cell construction for constant gas flow at atmospheric pressure are given elsewhere¹².

Identified overall electrode reactions can be represented by



H^+ transported through the electrolyte



By varying external cell resistance and monitoring the current (conversion rate) of the external circuit, polarisation curves can be obtained as shown in Fig. 1 for cells with platinum and ruthenium cathodes and perchloric acid electrolyte. Gas was fed continuously at both electrodes. The open circuit potential of ~ 0.93 V is reminiscent of that for a hydrogen–oxygen fuel cell and may reflect dissociative adsorption at the cathode to give N_{ads} and surface oxygen^{4,13,14} or possibly is due to the intermediate reaction



HNO intermediates have been postulated to decompose to give nitrous oxide and to be reduced by an alternate route to ammonia⁶ or hydroxylamine^{2–5}.

With some polarisation runs, product analyses were obtained for constant current over several hours. Gas chromatographic (GC) analyses for NO, N_2O and N_2 were done on cathode compartment (volume = 0.78 cm^3) outlet and inlet streams on Porapak Q columns. Ammonia and minor amounts of hydroxylamine accumulating in the electrolyte were measured using standard colorimetric methods^{15,16}. Most material balances on products accounted for generated current within 10% or better.

Some results for platinum and ruthenium cathodes (3.88 cm^2 apparent area) at constant current are shown in Table 1. The platinum black electrode was the commercial, American Cyanamid, Teflon-backed LAA-2 type^{17,18} (9 mg cm^{-2}). The ruthenium black electrode (9 mg cm^{-2}) was an AA-2 (refs 19, 20) type mechanically backed with porous Teflon. Possibilities of employing the electrogenerative cell as a chemical reactor are illustrated by Run 1 with platinum where the majority of the feed is converted to nitrous oxide with little ammonia formation. The effect of nitric oxide flow rate variation at lower potential is illustrated by Runs 3 and 4 on platinum where either ammonia or N_2O can predominate. This can be rationalised by strongly adsorbed nitric oxide^{7–9} displacement of surface intermediates with faster flow rates to give less reduced species and longer surface contact time at slower flows to produce more reduced species. The lower potential further favours ammonia formation. In Run 5, sulphuric acid electrolyte with platinum did not give results significantly different from perchloric acid. Results for NO reduction on ruthenium illustrate electrocatalytic specificity towards ammonia formation. Its unusual adsorptive properties for nitrogen and reduced intermediate species^{7,8,21} diminish the dependence of ammonia formation on flow rate.

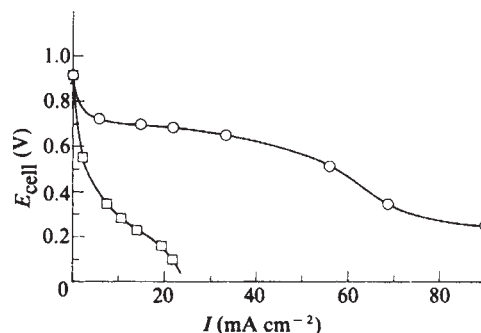


Fig. 1 Current density–voltage curve for electrogenerative reduction of nitric oxide at 25°C . IR corrected, platinum black anode, 2M HClO_4 aqueous electrolyte, 0.6 cm thick, $R_{\text{int}} = 0.39 \Omega$; $\circ$, Pt black; $\square$, Ru black cathode. NO feed $4.6 \text{ cm}^3 \text{ min}^{-1}$. (See text for details.)

Table 1 Nitric oxide–hydrogen electrogenerative cell operational data

Expt	Cathode**	Electro-lyte*	NO (cathode) feed rate, (cm ³ min ⁻¹)	Cathode potential† (V)	Current density (mA cm ⁻²)	Fraction of total current‡ (%)				Calculated§ coulombic efficiency	NO conversion (%)
						N ₂ O	N ₂	NH ₂ OH	NH ₃		
1	Pt	C	3.6	0.56	52	98.3	0.0	0.0	1.7	96.2	83
2	Pt	C	1.6	0.50	26	78.4	20.3	0.0	1.3	94.4	97
3	Pt	C	3.8	0.21	84	57.8	20.8	1.1	20.4	98.4	93
4	Pt	C	1.5	0.22	63	18.4	24.7	1.8	55.2	95.2	99
5	Pt	S	1.8	0.27	80	14.9	21.6	1.8	61.7	88.0	96
6	Ru	C	3.8	0.23	12	21.1	4.8	0.0	74.1	100.5	10
7	Ru	C	1.6	0.23	14	21.1	0.4	3.8	74.8	93.5	23
8	Ru	C	1.6	0.10	28	7.3	3.7	2.6	86.5	102.2	34
9	Pt	S	0.08	0.12	24¶	0	0	0	13.5	100.3	>99.5*

* C = 2M/HClO₄, S = 3M/H₂SO₄.

† Relative to hydrogen electrode.

‡ Normalised to 100%.

§ Based on generated current; analysis of cathode gas streams and electrolyte.

|| Overall feed rate is 4.5 cm³ min⁻¹, mixture contains 1.9% NO, 8.6% O₂ in N₂.¶ Involves substantial O₂ reduction.

* Final NO concentration in product gas stream was less than 100 p.p.m. (limit of GC detectability) after single pass through cell.

** Pt black anode, see text.

Ammonia formation favoured by ruthenium makes electro-generative reactor use attractive in conjunction with thermal conversion of nitrogen and oxygen to NO (ref. 22) to produce ammonia.

Reductions here take place at generated potentials positive to the hydrogen electrode as compared with the frequent cathodic potentials of conventional electrochemical reductions. With reactants separated in contrast to heterogeneous catalytic reductions, reactant competition for adsorptive sites is minimised, allowing thermodynamic factors to operate across the interface to favour reaction. One consequence is the controlled reaction at mild, room temperature conditions. Possibilities for reacting other nitrogen oxides as well as nitrogen and hydrogen to form ammonia in the electrogenerative mode are suggested by these results. The nitrogen–hydrogen reaction in particular may be favoured through minimisation of adsorptive competition.

The high nitric oxide conversion with platinum cathodes raises the possibility of using electrogenerative reactors as a means for NO_x pollution control at stationary power plants. To pursue this, a cathode feed mix of 1.9% nitric oxide and 8.6% oxygen with nitrogen was used with the cell of previous runs in Run 9, where nitric oxide concentration was reduced to less than 100 p.p.m. (0.01%) in a single pass in the presence of oxygen. The high NO conversion involved NO oxidation to NO₂ in the feed stream as well as electrogenerative reduction of NO₂ and remaining NO to ammonia, combined with current generating oxygen reduction to water. Nevertheless, preferential NO reduction in the presence of oxygen should be noted. With the cell at open circuit, NO concentration was not reduced below 1,100 p.p.m. by O₂ oxidation even further downstream. The reduction in NO concentration with the electrogenerative cell is significant in view of present environmental concern over NO_x pollutants²³. Investigations of straight NO electrogenerative reductions as well as simulated pollutant mixtures are continuing.

We thank the University of Wisconsin, the NSF and Exxon for support as well as Robert Pesselman of the Mill Street Foundation for experimental assistance. Any patent rights are assigned to the Wisconsin Alumni Research Foundation.

Received 26 October 1979; accepted 6 February 1980.

- Basolo, F. & Burwell, R. L. Jr (eds) *Catalysis: Progress in Research*, 153, 158, 188 (Plenum, New York, 1973).
- Snider, B. G. & Johnson, D. C. *Analyst. chim. Acta* **105**, 9–23 (1979).
- Jansen, L. J. J., Pieterse, M. M. J. & Barendrecht, E. *Electrochim. Acta* **22**, 27–30 (1977).
- Savodnik, N. N., Shepelin, V. A. & Zalkind, T. I. *Elektrokhimiya* **7**, 424–427 (1971).
- Savodnik, N. N., Shepelin, V. A. & Zalkind, T. I. *Chem. Abstr.* **75**, 14187 (1975).
- Kokes, R. J. *J. phys. Chem.* **70**, 296–297 (1970).
- Matson, S. L. & Harriot, P. *Ind. Engng Chem. Prod. Res. Dev.* **17**, 322–328 (1978).

- Voorhoeve, R. J. H. & Trimble, L. E. *J. Catal.* **38**, 80–91 (1975).
- Shelef, M. *Catal. Rev. Sci. Engng*, **11**, 1–40 (1975).
- Benson, R. E., Cairns, T. L. & Whitman, G. M. *J. Am. chem. Soc.* **78**, 4202–4205 (1956).
- Langer, S. H. & Landi, H. P. *J. Am. chem. Soc.* **86**, 4694–4698 (1964).
- Langer, S. H. & Sakellariopoulos, G. P. *J. electrochem. Soc.* **122**, 1619–1626 (1975).
- Low, M. J. D. & Taylor, H. A. *Can. J. Chem.* **37**, 544–549 (1954).
- Urabe, K., Aika, K. I. & Ozaki, A. *J. Catal.* **32**, 108–116 (1974).
- Kolthoff, I. M. & Sandell, E. B. *Quantitative Inorganic Analysis*, 668 (Macmillan, New York, 1948).
- Snell, F. D. & Snell, C. T. *Colorimetric Methods of Analysis* Vol. 4, 55 (Van Nostrand, New York, 1954).
- Landi, H. P. US Patent 3,407,096 (22 October 1968); US Patent 3,527,616 (8 September 1970).
- Voorhies, J. D., Mayell, J. S. & Landi, H. P. *Hydrocarbon Fuel Cell Technology* 455–464 (Academic, New York, 1965).
- Langer, S. H. & Landi, H. P. US Patent 3,248,267 (26 April 1966).
- Haldeman, R. G., Colman, W. P., Langer, S. H. & Barber, W. A. *Adv. Chem. Ser.* **47**, 106–115 (1965).
- Bond, G. C. *Catalysis by Metals*, 375 (Academic, New York, 1962).
- Gilbert, N. & Daniels, F. *Ind. Engng Chem.* **40**, 1719–1723 (1948).
- Ricci, L. J. *Chem. Engng* 33–36 (1977); 84–90 (1977); 70–76 (1977).

Leaching behaviour of rhyolite glass

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Glass has long been recognised as a potentially suitable matrix for diluting and stabilising nuclear waste^{1–5}. Most of the glasses which have been studied are borosilicates^{1–3}. One notable exception is the aluminosilicate nepheline syenite glass, used in the pioneering Canadian study⁴ that showed much lower apparent leach rates than for borosilicates^{4,5}. The natural aluminosilicate volcanic glass, rhyolite, has existed in some natural environments for millions of years^{6,7}; and, as Mendel has pointed out¹, it could be adopted as a model against which other glasses could be compared for nuclear waste disposal. We report here results on the hydrothermal leaching and recrystallisation of rhyolite at temperatures up to 300 °C, using not only the classical leach monitors of weight loss and solution analyses, but also modern surface probes such as SEM (scanning electron microscopy), ESCA (electron spectroscopy for chemical analyses) and SIMS (secondary ion mass spectroscopy). These surface techniques are especially valuable for gaining a more complete understanding of hydrothermal reactions of glasses or minerals^{8–10}.

The quaternary rhyolite sample from Mono Craters, California, was found to be homogeneous with the following composition (wt %): 4.2% Na₂O; 12.4% Al₂O₃; 76.9% SiO₂; 4.6% K₂O; 0.6% CaO; 0.8% FeO (total 99.5%). Either 0.2 g, 0.5 g or 1.3 g disks were drilled, cut, polished and cleaned ultrasonically in an acetone bath. The disks were placed in stainless steel autoclaves along with either (1) ~10 ml of distilled water (*pH* ~5.8), or (2) 5 g of crushed granite (composition 71.3% SiO₂; 14.4% Al₂O₃; 2.68% Fe₂O₃; 1.43% MgO; 1.68% CaO, 3.43% Na₂O; 3.95% K₂O; 0.07% MnO; 0.11% P₂O₅; 0.41% TiO₂)¹¹ plus 10 ml of distilled water. The autoclave was sealed with a stainless steel cone, and placed in an electrically heated Nichrome V tube furnace at temperatures of 100, 200, or 300 °C for up to 1 week.

The *pH* of the solution was recorded after cooling; and the aqueous phase was diluted to 50 ml with ~1% HCl. This solution was analysed for Na and Si using atomic absorption, and the glass disks were examined using the Philips 501 SEM, and ESCA 36 spectrometer, and the Applied Research Laboratories ion microprobe mass analyser at the Metaal Instituut TNO, Apeldoorn, Holland.

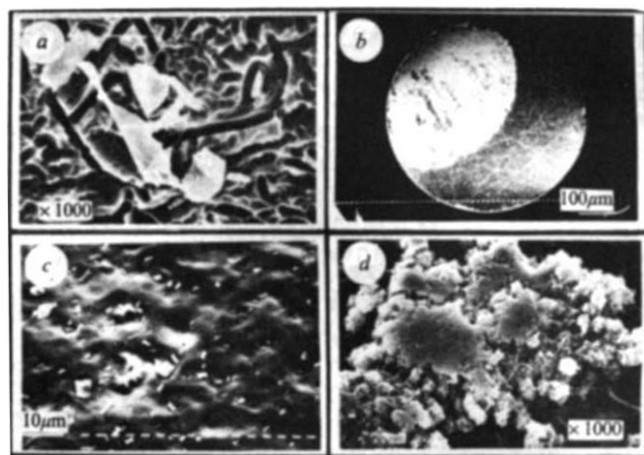


Fig. 1 Scanning electron micrographs of rhyolite glass after leaching with distilled water. *a*, Rhyolite at 200 °C, 3 days; *b*, rhyolite at 200 °C, 7 days; *c*, rhyolite + granite at 300 °C for 2 days; *d*, rhyolite at 300 °C, 3 days.

Table 1 Weight loss (%) of rhyolite disks after leaching in H₂O and granite plus H₂O*

Test condition	Leach time	<i>T</i> (°C)	% Weight loss
H ₂ O	1 hour	100	≤0.01
	2 hours	100	≤0.01
	6 hours	100	≤0.01
	1 day	100	0.01
	2 days	100	0.01
	3 days	100	0.05
	1 week	100	0.08
	1 day	200	<0.1
	3 days	200	0.9
	1 week	200	0.9
	1 day	300	0.16
	3 days	300	3.5
Granite plus H ₂ O	1 week	300	7.0
	1 week	100	<0.1
	1 day	200	<0.1
	3 days	200	0.63
	1 week	200	0.37
	6 hours	300	2.6
	3 days	300	23.5
	1 week	300	31.8

* The 100 °C experiments in distilled H₂O were performed with disks 1.2 cm in diameter and 0.5-cm thick, having a surface area of 4.1 cm² and weighing ~1.3 g. All other leaching experiments were performed with disks 8 mm in diameter and 2 mm thick having a surface area of 1.5 cm² and weighing ~0.2 g.

The weight losses for the leached rhyolite are given in Table 1. At 100 °C, there was very little weight loss, and the rhyolite leach rate at 100 °C of 3.6×10^{-5} g cm⁻² d⁻¹ (Table 2) was ~3–10 times less than those for borosilicate disks studied earlier^{2,5}. At 200 and 300 °C, there was an appreciable weight loss, and noticeable crystalline material appeared after three days of leaching in experiment (1). In the water-plus-granite experiment there was no noticeable crystalline material formed even after 1 week. The *pH* of the leachate increased slowly at 100 °C from 5.8 to 7.8, and the Na leached into solution from the 0.5 g sample increased approximately linearly with time at 100 °C (for example 3.1, 6.5 and 35.2 μg Na after 6 h, 1 day and 1 week leaching respectively).

The leach rates based on Na at 100 °C are slightly higher than the weight-loss leach rates (Table 2), indicating some selective leaching of Na. However, at 200 °C, the Na and weight loss leach rates are very similar, indicating congruent dissolution. At 300 °C, the lower Na leach rates indicate some selective reprecipitation of Na (see above). In recent laboratory studies at

120 °C, Zielinski¹² has also found that Si, K, and U in rhyolites are leached congruently, but previous work on natural weathered rhyolites (perlites) has usually indicated preferential leaching of sodium^{6,7}. Also, recent work by White and Claassen¹³ on rhyolite leaching at 25 °C, shows diffusion controlled parabolic kinetics, rather than the linear kinetics expected for congruent dissolution^{8,9}. Thus the mechanism of leaching probably changes between 25 °C and our higher temperatures.

From the weight losses at 200 and 300 °C for rhyolite (Table 1), and assuming a first-order dissolution process, an activation energy of ~12 kcal is readily calculated. This is very similar to that reported by Adams¹⁴ for rhyolite leaching below 100 °C, and substantially smaller than for borosilicates.

The scanning electron micrographs of the leached 0.2 g samples showed that at 100 °C no noticeable pitting or etching occurs in either experiments (1) or (2). At 200 °C, rather deep etch pits appear after a few days (Fig. 1*a*), and a crystalline product begins to cover the disk (Fig. 1*b*). This same product is produced in greater amounts in the 300 °C runs (Fig. 1*d*) and has been identified as muscovite by powder X-ray diffraction. The stability of muscovite at these temperatures is not surprising from Meyer and Hemley's laboratory observations¹⁵.

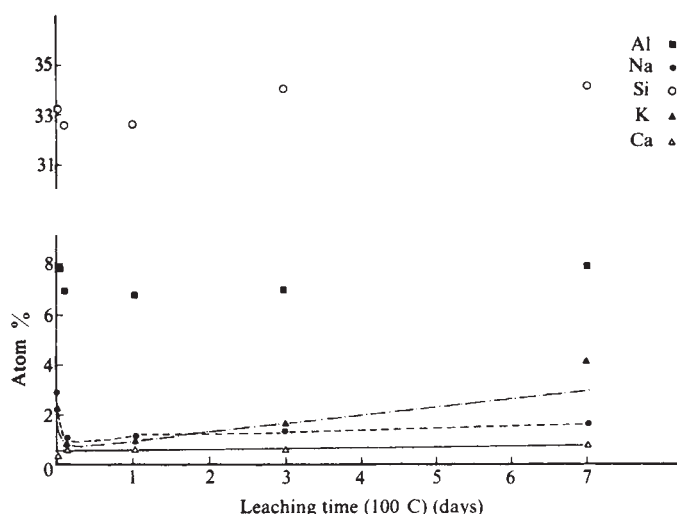


Fig. 2 ESCA surface analyses of the rhyolite disks leached in distilled water at 100 °C. The lines for Na, K and Ca are drawn only for clarity.

In contrast with the formation of large amounts of muscovite in distilled water, the glass disks reacted in the presence of granite (Fig. 1c) showed only a small amount of crystalline product which, based on the morphology of the crystals, appears to be feldspar. There must be a precipitation of aluminosilicates on the crystalline phases of the granite, which may be similar to the well-known amorphous silica reprecipitation¹⁶. This reaction is now under more detailed study. Our results, together with those of McCarthy *et al.*¹⁷, suggest that more work is needed on glass dissolution in the presence of complex rock matrices.

Table 2 Leach rates in g(glass) per cm⁻² per day⁻¹*

	T (°C)	Sample weight (g)	Leach rate	
			Weight loss	Na
Na borosilicates	100		$\geq 9 \times 10^{-5} \dagger$	—
			$3.5 \times 10^{-4} \ddagger$	—
Rhyolite	100	1.3	$3.6 \times 10^{-5} \S$	ND
		0.5	$3.3 \times 10^{-5} \S$	5.4×10^{-5}
	200	0.2	$1.7 \times 10^{-4} \S$	ND
		0.5	$4.2 \times 10^{-4} \S$	4.4×10^{-4}
		0.5	$3.7 \times 10^{-4} \S$	4.8×10^{-4}
	300	0.2	$1.3 \times 10^{-3} \S$	4.8×10^{-4}
		0.5	$1.3 \times 10^{-3} \S$	8.6×10^{-4}

* All leach rates are based on 1 week data. The Na leach rates were calculated assuming all of the glass constituents leached at the same rate as sodium (congruent dissolution).

† Ref. 5. ‡ Ref. 2. § From Table 1.

The ESCA and SIMS measurements complement the above observations, and show that rhyolite has hitherto unobserved surface leaching properties for a glass. From the areas of the appropriate ESCA peaks (Na KLL Auger, K 2p, Al 2p, Si 2p, Ca 2p and O 1s), the glass surface (≤ 50 Å) is readily analysed¹⁸⁻²⁰. The ESCA surface analyses of the unleached samples in atom. % are in semiquantitative agreement with the bulk probe analysis Bulk (and ESCA) analyses in atom. % are: Na; 2.8 (2.9); K; 2.0 (2.2); Al; 5.0 (7.9); Si; 26.5 (33.2); Ca; 0.2 (0.4); O; 63.3 (53.4). At 100 °C, Na and K are indeed leached preferentially from the surface (Fig. 2), as was indicated by the Na leach rates (Table 2). However, on further leaching, at 100 °C, the Na and K surface concentrations increase and approach the unleached values. Moreover, for all samples leached at 200 and 300 °C (1 hour to 1 week), all elemental surface concentrations are the same (within $\pm 10\%$) of the unleached sample. Congruent dissolution is the major mechanism of leaching in these experiments, as was indicated by the leach rates in Table 2.

Further confirmation of the congruent leaching mechanism comes from the SIMS measurements on unleached samples and samples leached at 100 and 300 °C. The leached samples show very similar concentration profiles to the unleached samples. All samples show very little concentration gradient for any of the elements measured; and in the unleached sample and the sample leached at 300 °C, Na and K are actually slightly enriched at the surface. All other SIMS measurements (ref. 8 and N. S. McIntyre, unpublished data) on other glasses have indicated a sharp decrease in surface alkali content after leaching.

We conclude that rhyolite is much more stable to leaching than are the borosilicates previously studied. Moreover, above 100 °C, the SEM, ESCA and SIMS results give no evidence for a surface depleted layer, as has been shown for many other glasses. Even without this layer, the glass is comparatively stable. Crystallisation of muscovite or clay minerals¹² will serve as useful sorbents for leached ions. More research is needed on such aluminosilicate glasses to understand fully the mechanism of dissolution and to minimise the temperature of formation while retaining their high leach stability.

We thank AECL (Canada) for financial assistance, and G. G. Strathdee, J. Tait, P. Taylor, R. Zielinski and H. Claassen for helpful comments and for preprints.

Received 10 December 1979; received 11 February 1980.

- Mendel, J. E. *Batelle Pacific Northwest Lab. Rep.* PN2-2764 (December 1978).
- Morris, J. B. *et al. Nature* **273**, 215-216 (1978).
- Int. Symp. Ceramics in Nuclear Waste Management*, Abstr. Session VI (American Ceramic Society, Cincinnati, 1978).
- Merritt, W. F. *Proc. Symp. Management of Radioactive Wastes from Nuclear Fuel Cycle Vol. 2*, 27-35 (IAEA, Vienna 1976).
- Strathdee, G. G., McIntyre, N. S. & Taylor, P. *Proc. Int. Symp. Ceramics in Nuclear Waste Management*, Cincinnati, CONF-790420 243-247 (US Department of Energy, Technical Information Center, Oakridge, 1979).
- Zielinski, R. A., Lipman, P. W. & Millard, H. T. Jr *Am. Miner.* **62**, 426-437 (1977).
- Lipman, P. W., Christiansen, R. L. & Van Alstine, R. E. *Am. Miner.* **54**, 286-291 (1969).
- Hench, L. L. & Clark, D. E. *J. Non-Cryst. Solids* **20**, 83-105 (1978).
- Holdren, G. R. Jr & Berner, R. A. *Geochim. cosmochim. Acta* **43**, 1161-1171 (1979).
- Bird, G. W., Fung, P. & McIntyre, N. S. *7th Can. Seminar Surfaces*, Pinawa, Canada, (1979).
- Fyfe, W. S., Kronberg, B. I. & Leonards, O. H. *Soil Sci. Pl. Nutr.* **24**, 455-464 (1978).
- Zielinski, R. A. *Chem. Geol.* **27**, 47-63 (1979).
- White, A. F. & Claassen, H. C. *Chem. Geol.* **28**, 91-109 (1980).
- Adams, P. B. *Proc. Int. Symp. Ceramics in Nuclear Waste Management*, Cincinnati, CONF-790420 233-237 (US Department of Energy, Technical Information Center, Oakridge, 1979).
- Meyer, C. & Hemley, J. J. in *Geochemistry of Hydrothermal Ore Deposits* (ed. Barnes, H. L.), 212-215 (Holt, Rinehart and Winston, New York 1967).
- Fyfe, W. S., Price, N. J. & Thompson, A. B. *Fluids in the Earth's Crust*, 75, 97 (Elsevier, Amsterdam 1978).
- McCarthy, G. J. *et al. Nature* **273**, 216-217 (1978).
- Wagner, C. D. in *Quantitative Surface Analysis of Materials ASTM STP 643*, 31-46 (ed. McIntyre, N. S.) (American Society for Testing and Materials, 1978).
- Adams, J. M., Evans, S., Reid, P. I., Thomas, J. M. & Walters, M. J. *Analyt. Chem.* **49**, 2001-2008 (1977).
- Bancroft, G. M., Gupta, R. P., Hardin, A. A. & Ternan, M. *Analyt. Chem.* **51**, 2102-2107 (1979).

Winds in the thermosphere of the Northern Polar Cap

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A preliminary report is presented here of a sequence of measurements of the thermospheric wind in the Northern Polar Cap covering nine complete periods of 24 h. This unique data set covers all local times and a range of magnetic activities with K_p values from 0 to 6⁻. Certain major features of the wind pattern were found to be independent of magnetic activity: night-time winds were steady and approximately antisolar, whereas in the morning and evening sectors the wind weakened and the direction became variable as if the observation point passed through a vortex. On the dayside, the wind was northwestward in quiet conditions but tended towards the antisolar direction with increasing activity. The simplicity of the wind pattern in geomagnetic coordinates and its similarity to the published maps of ion drift strongly suggest that the momentum of the neutral gas is supplied from active ion drag and hence the energy of circulation comes from the magnetospheric electric field.

A ground-based optical Doppler technique was used and the observations were made at Longyearbyen (geographic coordinates 78.2° N, 15.6° E) during January 1979. At that time of the year there is unrelieved darkness for the whole day which permitted the continuous observation of the aurora. Although discrete forms were frequently seen in localised areas of the sky, for the most part, observations were being made on the diffuse background which was of subvisual intensity. Measurements of the oxygen emission at 630 nm in the aurora were used to find the wind velocity at a height of ~230 km. Using a passively stabilised scanning Fabry-Perot interferometer determinations of the horizontal components of the neutral wind were made with a typical error bar of 20 m s⁻¹. The maximum speed frequently occurred between 2100 and 0300 MLT (magnetic local time) and was directed towards the Equator. During these observations, the highest speed observed was 450 m s⁻¹ which

occurred at 2400 h on the 27 January 1979 when the magnetic character figure K_p was 4^+ . On less active periods the wind at 2400 h was smaller; for example, on 21 January 1979, when $K_p = 0$, it was 100 m s^{-1} .

In Fig. 1a, a 24-h plot in geomagnetic coordinates shows the wind vectors at 15-min intervals drawn with their tails on the circle which is the locus of the position of Longyearbyen as the Earth rotates. The predominance of the antisolar wind is evident except for a few hours near 0600 and 1700 h magnetic time when the wind weakens which suggests that there are local vortices in the vicinity. The major region of cross-polar flow seems to lie poleward of 78.2°N during the morning and afternoon periods. The value of the magnetic character figure A_p for this day was 23. The wind pattern for a less active day, 21 January 1979, when A_p was 11, is shown in Fig. 1b. Although the antisolar flow clearly dominates the nightside, it is mixed with a westward component on the dayside. This builds up from early morning, maximising in the afternoon and diminishing in early evening before the onset of the equatorward wind. Such behaviour is more typical of lower geomagnetic activity, and was also observed on the 9, 12, 20 and 22 January when A_p was 15, 10, 12, and 14 respectively. Other examples of the same type as in Fig. 1a were found on 24 and 25 January when A_p was 23 and 34 respectively.

Reports of rocket-borne experiments flown at high latitudes within the Polar Cap¹⁻³ have also shown, for the limited duration of the vapour trail, the existence of the antisolar wind. The occurrence of the westward wind on the dayside is a feature of less active days when one may expect that the auroral oval has

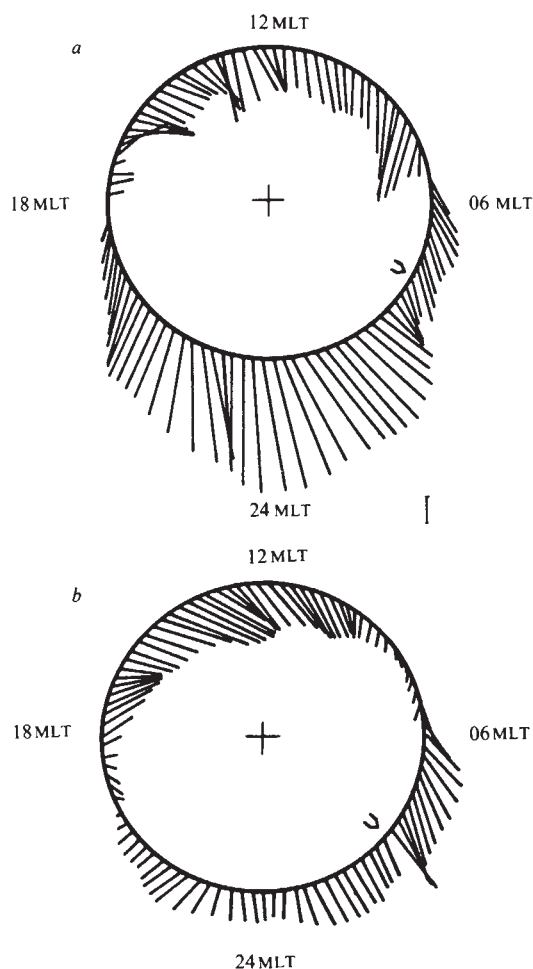


Fig. 1 A 24-h plot in geomagnetic coordinates showing vectors representing the neutral wind in the polar thermosphere during: a, 27 January 1979. b, 21 January 1979. The circle is at a geomagnetic latitude of 73.1°N , traced out by the observing site during the period of observation. The vectors are drawn with their tails on the circle. Scale bar, $100 \text{ m s}^{-1} \text{ MLT}$.

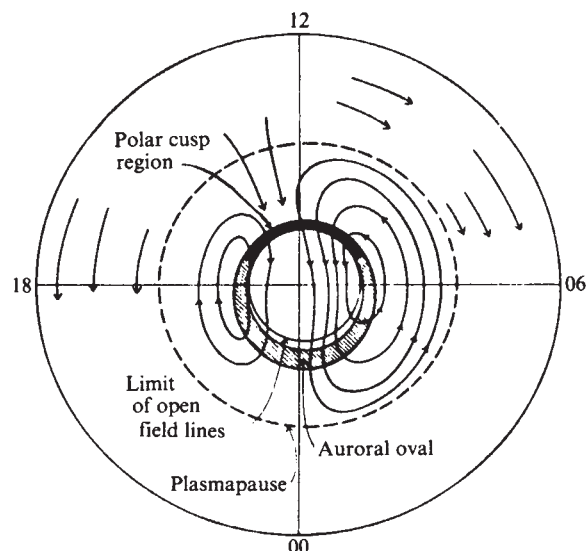


Fig. 2 A qualitative view of global winds including the effects of convection electric fields. Figure 13 from Fedder and Banks⁵ redrawn to make a direct comparison with Fig. 1.

contracted and the magnetospheric cusp lies on magnetic field lines to the north of Longyearbyen. In these conditions, the ionosphere above the observatory site is in a region of weak penetration of the magnetospheric electric field and, being in shadow from the solar EUV radiation, has a low ion concentration. The ion momentum source being weakened, the influence of the global scale pressure distribution may have greater significance. Note in this connection, that the geomagnetic and geographic frames of reference at Longyearbyen differ in azimuth by $\sim 45^\circ$. In consequence, when the data are presented in geographic coordinates, these daytime winds are within a few degrees of westward. A possible mechanism, not involving ions, would require a large scale pressure gradient with pressure increasing towards the Pole which, combined with the Coriolis effect, would drive a westward wind. However, the 0600 data on exospheric temperatures showed a broad temperature minimum at the Pole at the winter solstice⁴ which is not consistent with the present interpretation.

The general features of a high latitude neutral wind pattern dominated by ion convection are shown in Fig. 2 as given by Fedder and Banks⁵ and are also shown by the present results. The cross-polar antisolar flow arises from the dusk to dawn magnetospheric electric field. Vortices in the morning and evening occur where the sunward convection in the auroral zone causes the neutral wind to reverse direction as latitude decreases. Some workers conclude that winds at high latitudes up to the auroral zone are very much dependent on pressure gradients arising due to Joule heating⁶. However, the major features of the thermospheric wind in the Polar Cap seem to be consistent with the theory that most of the momentum is derived directly from the convecting ions⁷ which transfer energy stored in the magnetospheric electric field into kinetic energy of circulation by active Hall drag. The results presented here show that more energy is transferred with increasing magnetic activity and indicate that a greater latitudinal extent of the thermosphere is included in the circulation with greater A_p .

Received 31 December 1979; accepted 27 February 1980.

- Meriwether, J. W., Heppner, J. P., Stolarik, J. D., Westcott, E. M. *J. geophys. Res.* **78**, 6643-6661 (1973).
- Kelly, M. C., Stockflet Jorgensen, T. & Steen Mikkelsen, I. *J. atmos. terr. Phys.* **39**, 211-219 (1977).
- Westcott, E. M., Stenbaek-Nielsen, H. C., Davis, T. N., Jefferies, R. A., & Roach, W. H. *J. geophys. Res.* **83**, 1565-1575 (1978).
- Thuiller, G., Falin, J. L. & Barlier, F. *J. atmos. terr. Phys.* **39**, 1195-1202 (1977).
- Fedder, J. A. & Banks, P. M. *J. geophys. Res.* **77**, 2328-2340 (1972).
- Rothwell, P., Mountford, R. & Martelli, G. *J. atmos. terr. Phys.* **36**, 1915-1926 (1974).
- Mayr, H. G. & Harris, I. *J. geophys. Res.* **83**, 3327-3336 (1978).

Phytoplankton growth and zooplankton grazing in oligotrophic oceans

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Central oceanic regions such as the Sargasso Sea and the North Pacific Gyre have traditionally been thought of as biological deserts^{1,2}. New phytoplankton production estimates³ and interpretations of physiological data^{4,5} have suggested that these areas are, in fact, highly productive and that nutrient exchanges supporting these high growth rates are extremely transient. Fast phytoplankton growth rates must be balanced by equally fast zooplankton-caused mortality. Zooplankton abundances and their filtration rates, however, are consistent with slow phytoplankton growth rates, not fast. It is shown here that the dominant grazers in oligotrophic areas seem to be microzooplankton, and that molecular diffusion limits the effect of nutrient pulses, implying that bulk nutrient concentrations are most important. Present growth studies imply that phytoplankton can meet their growth needs with nanomolar ammonia concentrations.

Ambient concentrations of such nitrogenous nutrients as ammonia are below detection limits with conventional chemical methods in surface waters of central oceanic oligotrophic areas^{6,7}. Indirect evidence suggests that the concentrations of ammonia and urea in the North Pacific central gyre⁷ are less than 50 nM. This is far lower than average values for unpolluted coastal waters^{8,9}, and very low compared with half-saturation concentrations for most phytoplankton grown in cultures¹⁰.

Growth rates for phytoplankton growing with steady-state nutrient kinetics at such low concentrations would be low, a fact supported by ¹⁴C incubation measurements in the central North Pacific Gyre. Average specific growth rate, calculated as carbon assimilation rate divided by living carbon concentration⁷, is about 0.1 per day.

Phytoplankton growth measured by other techniques can yield much higher rates. The shortest doubling time reported is 3 h in the Sargasso Sea, calculated from ATP concentration changes in incubated water samples³. This is equivalent to a specific growth rate of 2.8 per day for a 12-h growth day.

Cellular nutrient status determines and can, therefore, indicate growth rate. Phytoplankton grown in cultures have their carbon, nitrogen and phosphorus contents in the same relative proportions as those in oceanic phytoplankton (in the Redfield ratio) only when growing at maximal rates⁵. If the physiology of oceanic phytoplankton is the same as that of

cultured phytoplankton then oceanic cells also grow at maximum rates⁵. Oceanic cells with the same maximum growth rate as cultured cells would grow at 2–3 per day.

These high growth rates cannot be supported for cells growing in a steady state with measured nitrogenous nutrient concentrations. Because slightly N-depleted phytoplankton display elevated uptake rates when compared with N-sufficient algae and because a source of regenerated nitrogen is zooplankton excretion, McCarthy and Goldman⁴ suggested that phytoplankton in oligotrophic oceans may obtain their nitrogen needs by rapid, transient uptake of excreted nitrogen. They noted such nutrients should be present at concentrations well above average in the wakes of swimming animals. They recognised that such elevated concentrations would persist only briefly. Thus, phytoplankton might appear to be growing successfully at the expense of virtually undetectable small-scale nutrient pulses.

The predominant source of phytoplankton mortality in low nutrient input regions such as mid-ocean gyres is zooplankton grazing. Calculation of grazing rates by multiplying typical laboratory-measured filtration rates and field-measured grazer densities (Table 1) shows that microzooplankton, especially protozoa, are dominant herbivores in the loss to grazers of 11% per day (0.11 per day) in the oligotrophic Pacific.

The conclusion that microzooplankton in the North Pacific Gyre are the dominant grazers explains the low rate of zooplankton excretion measured there¹¹. Specific regeneration rate of the macrozooplankton, calculated by dividing 2.4 nM per day nitrogen regeneration rate¹¹ by the 0.2 µM particulate nitrogen concentrations, is 0.012 per day. Microzooplankton feeding at a total filtration rate 5.4 times as fast (Table 1) will increase this rate to 0.007 per day.

Continuous, steady state uptake has specific growth rates equalling specific uptake rates. To achieve a 0.1 per day growth rate, concentrations less than now measurable would be adequate. For example, *Thalassiosira pseudonana* growth rate is 2.6 per day (81% of maximum) for ammonia concentrations at the detection limit, 0.1 µM (refs 22, 23). If growth follows the Monod equation,

$$\mu = \mu_{\max} \frac{C}{C + K_s} \quad (1)$$

with μ the specific growth rate, $\mu_{\max}$ the maximum growth rate, C the ammonia concentration, and K_s the half-saturation concentration, then a K_s value of 23.5 nM can be calculated from the above concentration and growth rate. Such a cell could maintain a growth rate of 0.1 per day at an ammonia concentration of 0.8 nM. A growth rate of 2.6 per day would, of course, require 0.1 µM ammonia.

Short term non-steady state ammonia uptake rates can be much faster than growth rates: instantaneous ammonia uptake rates are as high as 15 per day, equivalent to doubling times of 0.05 days or 4.3×10^3 s (ref. 4). Phytoplankton cells would have to take up nitrogen at maximum capacity for this length of time to accumulate enough nitrogen to support one cell division.

Table 1 Biological scales for central N. Pacific Gyre region

	Phytoplankton	Protozoa	Microzooplankton Nauplii	Post-nauplii	Macrozooplankton Copepods	Thaliaceans
Concentrations (C) (individuals per litre)	600,000*	655*	3*	1*	0.1†	$1 \times 10^{-4} \ddagger$
Separation distance (D) (cm)	0.1	1.1	5	10	21	—
Swimming speed (S) (m per day)	—	120§	—	—	1,200	—
Daily path separation (cm)	—	0.01	—	—	0.29	0
Individual filtration rates (ml per individual per day)	—	0.120¶	2#	10#	168**	500††
Total filtration rate (ml per litre per day)	—	75	6	10	16.8	0.05

Total filtration rate is 108 ml per litre per day, equal to a 0.11 per day grazing rate. Separation distance is $C^{-1/3}$. Daily path separation assumes all swimming paths are parallel and equally spaced, $(C^{-1} S^{-1})^{1/2}$.

* Ref. 17; † M. M. Mullin, personal communication; ‡ doliolids are dominant Thaliaceans (J. A. McGowan, personal communication); § ref. 18; || ref. 19; ¶ ref. 15; # ref. 20; ** ref. 21; †† Filtration rate is that of *Doliolletta gegenbaui* (D. Deibel, personal communication).

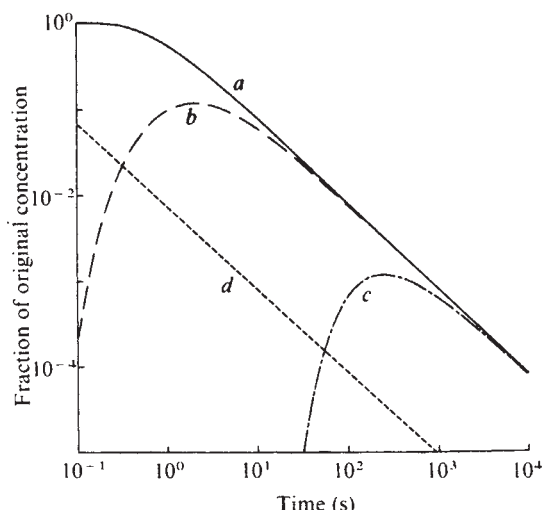


Fig. 1 Concentrations through time after a plume is created. Concentrations are fractions of original plume concentration. *a*, *b*, *c* show the effect of a 100 μm wide plume which is representative of a copepod's passage. *a*, At centre of plume; *b*, 100 μm from plume centre, 50 μm from initial plume edge; *c*, 1,000 μm from plume centre; *d*, effect of 10 μm wide plume representative of protozoa passage at centre of plume.

Phytoplankton growing at 0.1 per day could meet their nitrogen needs at uptake rates 1% of the maximum 15 per day.

A discontinuous nutrient source would be excreta from animals swimming near a phytoplankter. From the moment the animal excretes them, nutrients diffuse away. A phytoplankter will see a different range of nutrient concentrations depending on where it is relative to the plume. The concentration in a plume with square cross-section can be easily calculated. The relative concentration, f , at a given time after excretion, t , at a distance away from the centre along the centre line, x , for a plume of initial width, $2a$, and diffusivity, D ($= 10^{-5} \text{ cm}^2 \text{ s}^{-1}$),

$$f = \frac{1}{2} \left\{ \text{erf} \left(\frac{a-x}{2(Dt)^{1/2}} \right) + \text{erf} \left(\frac{a+x}{2(Dt)^{1/2}} \right) \right\} \times \text{erf} \left(\frac{a}{2(Dt)^{1/2}} \right) \quad (2)$$

where $\text{erf}(z)$ is the error function of z (ref. 12, p. 56).

The results (Fig. 1) show that even within the plume, nutrient concentrations drop rapidly. Three hundred seconds after a copepod-sized pulse (100 μm across) is released, concentrations have dropped by three orders of magnitude at the pulse centre and are a maximum 0.1 cm away that is $<0.001\%$ of the initial concentration. Molecular diffusion away from protozoan wake will be faster. Concentration at the centre of a 10 μm plume will have dropped to 0.1% of the initial concentration within 0.1 s. Because they will be further from the pulse most phytoplankton will see even lower concentrations.

Ammonia concentration in a plume behind a swimming herbivore can be calculated several ways. A grazer could be viewed as converting the particulate nitrogen in the water it filters to ammonia. By continually converting the 0.2 μM particulate nitrogen⁷ to ammonia, it would leave a trail of 0.2 μM ammonia. This supposes continual conversion. Sporadic excretion would raise concentration in a given pulse but decrease water volume affected. McCarthy and Goldman⁴ calculated that such a sporadic pulse could contain 5 μM ammonia around a copepod. Thus, the maximum nutrient concentration behind a protozoan will have decreased from 200 to 2 nM within 1 s; the same concentration change behind a copepod would occur within 100 s. These times are much shorter than the 4,300 s earlier calculated as the length of time required to accumulate sufficient N for a doubling. Individual nutrient pulses are not sufficient to be the main nitrogen source for phytoplankton.

These calculations neglect concentration decreases caused by nutrient uptake and assume that only molecular diffusion is important. Phytoplankton and turbulence cause concentrations to decrease faster than has been calculated here.

Usefulness of these molecular diffusion calculations depends on nutrient recycling occurring on small scales. Comparison of feeding rates for potential oceanic herbivores shows that small grazers—protozoa and copepods—dominate the larger thaliaceans, accounting for 99% of water filtered. The marine copepod *Paracalanus parvus* incorporates 37% of ingested particulate nitrogen and recycles the rest¹³. If mid-oceanic grazers recycle at the same 63%, then microzooplankton and copepods recycle at least 62% of ingested primary production for uptake by surviving phytoplankton, either directly or through bacteria. Thus, nitrogen cycling occurs predominantly on the microscale. Inputs on a larger scale, from salp aggregates or fish schools, may be more visible but less important.

Importance of nutrient interactions on a small scale is analytically convenient because diffusion-based mixing processes are so much better understood than those based on turbulence. Additionally, the complete range of phytoplankton-herbivore interactions would be occurring at any given time in the medium scale water sample. This would imply that the mean nutrient concentration through time at a given phytoplankter (that is, what a cell sees) would be the same as the mean concentration through space at a given time (that is, a water sample). A linear uptake-ammonia relation would imply that mean nutrient uptake rate is linearly related to the mean concentration through time at the cell, which is in turn linearly related to the measured concentration. As long as uptake rate is a linear function of ammonia concentration, the amount of ammonia will provide a useful mirror of an individual phytoplankter's environment. Less than linear uptake rates, indicative of saturation of cellular systems, would cause a linear model to overestimate cellular nutritional status.

Microzooplankton, which control N cycling in the temporally and spatially uniform central North Pacific Gyre¹⁴, need not dominate elsewhere: in the very heterogeneous coastal California protozoa are relatively unimportant¹⁵. The northern Sargasso Sea, where cold core rings introduce variability not seen in the central Pacific¹⁶, may have different grazers. However, similarity in zooplankton volumes for the North Pacific and the North Sargasso Sea^{14,16} suggest that the two oligotrophic systems are similar.

Why, then, do oceanic phytoplankton show nutritional composition characteristic of nutritionally fit cells? If oceanic algae grow at a rate of 0.1 per day and if the relationship between cellular nutrient status and maximum growth rates found in culture studies⁵ apply to all phytoplankton, then algae from oligotrophic oceanic areas have lower maximum growth rates. This would imply that the algae have adapted to perpetually low growth rates by losing the ability to grow fast. Further understanding of nutrient cycling in these oligotrophic areas will depend on the development of techniques to measure these low concentrations.

This study was funded by the US Department of Energy on contract DE-AM03-76-SF00010. I thank T. Platt, J. Cullen, I. Heaney and especially R. Eppley, J. Beers, J. McGowan, D. Deibel and R. Armstrong for helpful suggestions.

Received 12 November 1979; accepted 13 February 1980.

1. Parsons, T. R., Takahashi, M. & Hargrave, B. in *Biological Oceanographic Processes* 2nd edn (Pergamon, Oxford, 1977).
2. Ryther, J. H. in *The Sea* Vol. 2 (ed. Hill, M. N.) 347–380 (Wiley, New York, 1963).
3. Sheldon, R. W. & Sutcliffe, W. H. *Limnol. Oceanogr.* **23**, 1051–1055 (1978).
4. McCarthy, J. J. & Goldman, J. C. *Science* **203**, 670–672 (1979).
5. Goldman, J. C., McCarthy, J. J. & Peavey, D. G. *Nature* **279**, 210–215 (1979).
6. Carpenter, E. J. & McCarthy, J. J. *Limnol. Oceanogr.* **20**, 389–401 (1975).
7. Eppley, R. W., Sharp, J. H., Renger, E. H., Perry, M. J. & Harrison, W. G. *Mar. Biol.* **39**, 111–120 (1977).
8. Eppley, R. W., Renger, E. H., Harrison, W. G. & Cullen, J. J. *Limnol. Oceanogr.* **24**, 495–509 (1979).
9. Walsh, J. J., Whitledge, T. E., Barvenik, F. W., Wirick, C. D. & Howe, S. D. *Limnol. Oceanogr.* **23**, 659–683 (1978).
10. Eppley, R. W., Rogers, J. N. & McCarthy, J. J. *Limnol. Oceanogr.* **14**, 912–920 (1969).

11. Mullin, M. M., Perry, M. J., Renger, E. H. & Evans, P. M. *Mar. Sci. Commun.* **1**, 1–13 (1975).
12. Carslaw, H. S. & Jaeger, J. C. in *Conduction of Heat in Solids* 2nd edn (Oxford University Press, 1959).
13. Checkley, D. M. thesis (University of California, San Diego, 1978).
14. McGowan, J. A. in *Oceanic Sound Scattering Prediction* (eds Anderson, N. R. & Zahuranc, B. J.) 423–443 (Plenum, 1977).
15. Heinbokel, J. F. *Mar. Biol.* **47**, 191–197 (1978).
16. Ortnier, P. B., Wiebe, P. H., Haury, L. & Boyd, S. *Fish. Bull.* **76**, 323–334 (1978).
17. Beers, J. R., Reid, F. M. H. & Stewart, G. L. *Int. Rev. ges. Hydrobiol.* **60**, 607–638 (1975).
18. Heinbokel, J. R. *Mar. Biol.* **47**, 177–189 (1978).
19. Enright, J. T. *Limnol. Oceanogr.* **22**, 118–125 (1977).
20. Mullin, M. M. & Brooke, E. R. in *Marine Food Chains* (ed. Steele, J. H.) 74–95 (Oliver and Boyd, Edinburgh, 1970).
21. Frost, B. W. *Limnol. Oceanogr.* **17**, 805–815 (1972).
22. Goldman, J. C. & McCarthy, J. J. *Limnol. Oceanogr.* **23**, 695–703 (1978).
23. Strickland, J. D. H. & Parsons, T. R. in *A Practical Handbook of Seawater Analysis* 2nd edn (Fisheries Research Building, Canada, 1972).

Stromatolites 3,400-Myr old from the Archean of Western Australia

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Internally laminated conical mounds characterise a regionally extensive chert unit near the top of the 3,400-Myr old Warrawoona Group in the Pilbara Block of Western Australia. The chert formed by silicification of a carbonate–evaporite sequence deposited in shallow subtidal to intertidal environments. The morphology and internal organisation of the mounds described here suggests that they are conical stromatolites similar but not identical to members of the common Proterozoic group *Conophyton* Maslov.

The Warrawoona Group of the eastern Pilbara region of Western Australia (Fig. 1) is a typical Archean greenstone belt volcanic sequence^{1,2}. The 10-km thick section consists largely of volcanic rocks interstratified with cherty sedimentary units generally <30 m thick. Low greenschist-grade metamorphism has affected the entire sequence, but, in the areas studied, shearing is largely absent. Cherty units commonly show only minor recrystallisation.

During sedimentological studies in the eastern Pilbara, I identified a distinctive stromatolitic chert unit near the top of the Warrawoona Group in at least three of the Pilbara structural belts (Fig. 1). Here this unit is called the Strelley Pool chert for outcrops at Strelley Pool (21°06'33"S, 119°08'14"E) in the Pilgangoora Syncline (Fig. 1). The unit averages a relatively constant 20–25 m in thickness, although it reaches 50 m on the

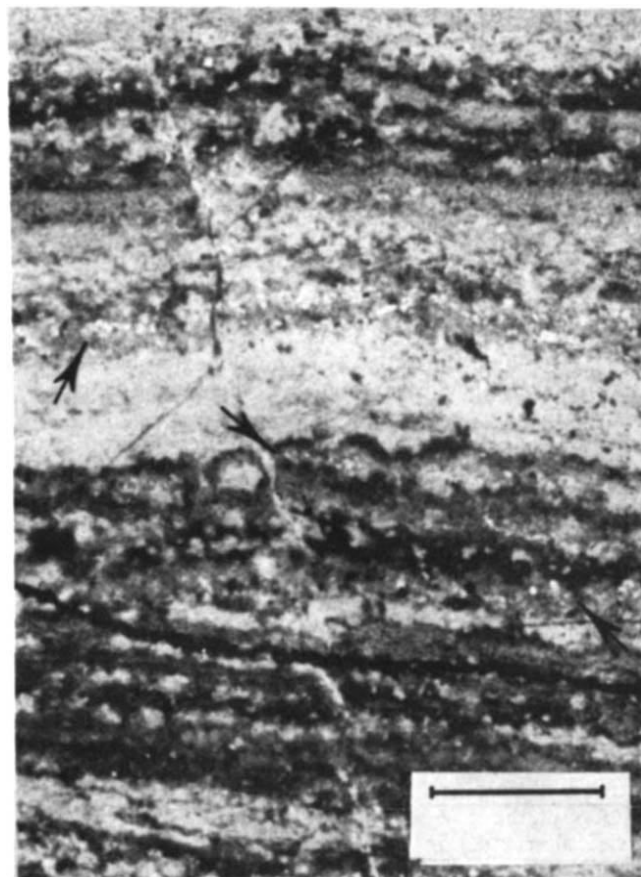


Fig. 2 Photomicrograph of alternating light and dark flat laminae from area between conical stromatolites. Arrows point to layers containing detrital quartz grains. Micromodular character of translucent laminae resembles fenestral texture of modern algal mats. Scale bar, 0.5 cm.

north flank of the North Pole Dome (21°00'43"S, 119°28'52"E). An excellent section in the Kelly Belt occurs near Spinaway Creek at 21°35'S, 120°01'01"E.

The Strelley Pool chert displays a nearly constant internal stratigraphy in the three structural belts in which it was identified. It can be subdivided into two main members. The lower 10–15 m consist largely of finely laminated chert. The rock shows thin, varve-like alternating laminae of light grey chert and dark grey carbonaceous and pyritic chert or of calcareous and non-calcareous chert (Fig. 2). The laminae may be flat and even but commonly form isolated or clustered conical mounds here interpreted to be stromatolites. This subdivision characteristically includes units up to 1 m thick composed of massive, coarsely crystalline silicified evaporite. These units are present in every section examined. In the Pilgangoora Syncline, a quartzite layer is present locally at the base of the chert, and thin laminae and wisps of detrital quartz occur irregularly in the overlying laminated chert (Fig. 2). Along at least two horizons, underlying laminae are truncated and cut by desiccation cracks filled with detrital sand, but, overall, coarse clastic layers, current structures, and evidence of exposure are rare. This subdivision was deposited in a subtidal to lower intertidal environment and originally consisted of interlayered evaporite and carbonate sediments.

The upper part of the Strelley Pool chert includes a variety of dark to light grey, white, and greenish cherts. Layers of large silicified evaporite crystals occur towards the base. Large pods of silicified collapse breccia and cavities containing silicified stalactitic and stalagmitic fill indicate the existence of considerably greater amounts of original evaporite and extensive post-depositional exposure, leaching, and solution collapse. Small intraformational conglomerates, scours, and other features

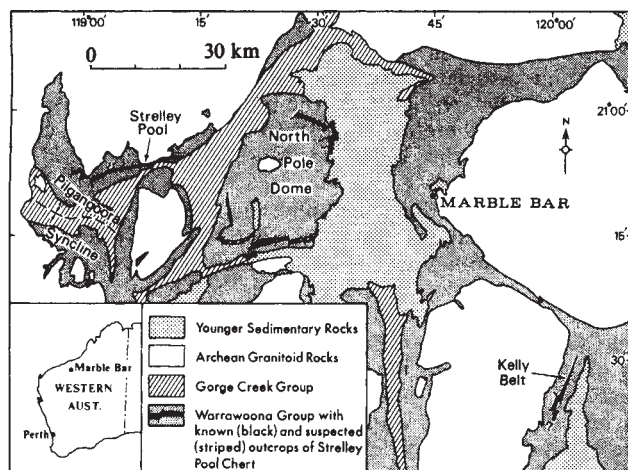


Fig. 1 General geological map of the northeastern Pilbara region showing known and suspected outcrops of the Strelley Pool chert.

suggest deposition in low energy environments ranging from shallow subtidal to intertidal. In the Pilgangoora Syncline, this unit is capped by detrital volcanoclastic material that may represent alluvial deposits.

The regional consistency of the detailed internal stratigraphy of the Strelley Pool chert across the entire 100+ km extent of its known outcrop (Fig. 1); its distinctive and regionally consistent sedimentology; and the characteristic development of stromatolites are unlike other chert units in the Warrawoona Group and indicate that the Strelley Pool chert is not correlative with better known chert units at Marble Bar and near the base of the North Pole stratigraphic sequence¹. These cherts lie several kilometres stratigraphically below the Strelley Pool chert. The latter represents a single, large, regionally extensive Archean evaporite basin which covered much of the area of the modern eastern Pilbara.

Unbranched, conical, columnar stromatolites occur within the lower subdivision of the Strelley Pool chert across its entire outcrop. They occur as distinct conical mounds with a relief generally between 2 and 6 cm (Figs 3 and 4). Individual mounds may extend vertically through as much as 0.6 m of rock. Apical angles average 70° to 80° (Fig. 4), but are locally as low as 30°. The internal lamination is fine and continuous, even across areas between widely spaced stromatolites. In horizontal sections and rare occurrences where the stromatolites show in relief on bedding surfaces, their shape varies from circular to elliptical (Fig. 3).

These structures are considered to be biogenic for several reasons. Their gross morphology and internal structuring are like those of post-Archean conical stromatolites, particularly members of the group *Conophyton* Maslov^{3,4}. Fine laminae showing alternating light and dark layers are characteristic of many Proterozoic and early Archean unbranched conical columnar stromatolites. However, the Strelley Pool stroma-



Fig. 4 Vertical slab through same block of stromatolitic chert shown in Fig. 3. Note slight axial thickening of laminae. Darker areas are calcareous; light areas are nearly pure chert.

tolites differ from true *Conophyton* Maslov in generally lacking a well defined axial zone. Individual laminae instead are continuous across the apical region, showing a slight thickening in most specimens (Fig. 4). Their occurrence in shallow subtidal evaporitic deposits in the Pilbara accords well with previous interpretations that conical stromatolites commonly develop in subtidal environments by alternating periods of organic growth and chemical precipitation^{5,6}. The continuity of the laminae from stromatolite mounds to flat inter-mound areas in the Strelley Pool chert indicates that large areas of the sea floor were covered by flat organic mats. In the Pilgangoora Syncline, the flat cryptogalaminites contain detrital sand grains and sand laminae (Fig. 2), features characteristic of both modern and ancient algal and bacterial mats reflecting their binding and trapping ability.

Lavas from the Duffer Formation near the middle of the Warrawoona Group have been dated at about 3,450–3,490 Myr BP (refs 8, 9). These rocks are 1–2 km stratigraphically below the Strelley Pool chert, and, although the possibility of intervening unconformities cannot be dismissed, the overall igneous and sedimentological continuity of the Group suggests that major hiatuses are absent. Following deposition of the uppermost part of the Warrawoona Group and the suprajacent Gorge Creek sediments, the rocks were extensively deformed and intruded by granitoid plutons. The main period of deformation and associated plutonism has been dated at about 2,900–3,100 Myr (refs 10, 11). The possible age range for the stromatolites is, therefore, 3,450–3,100 Myr, but their close association with the older rocks suggests deposition at ~3,400 Myr.

The importance of the stromatolites in the Strelley Pool chert lies in their implications towards the nature of early life forms. Archean stromatolites have been described previously from the 2,600–2,700 Myr-old rocks in Canada^{12,13}, from the 2,500–2,800 Myr-old Bulawayan Group in Rhodesia^{14,15}, and from the 3,000 Myr-old Pongola Supergroup in South Africa¹⁶. Dunlop and others¹⁷ have previously reported oncolitic and mat-like structures which they interpret to be stromatolites from the Warrawoona Group, but they are apparently of local occurrence, do not show a well developed mound-like habit, and have not been documented as being biogenic.

The Strelley Pool stromatolites are similar in morphology and internal structure to stromatolites from younger Archean and Proterozoic sequences. If these and other Archean stromatolites



Fig. 3 Surface morphology of conical stromatolites in the Strelley Pool chert. Note elliptical shape of many of the larger cones. This specimen from outcrops west of Strelley Pool at 21°08'S, 119°00'28"E. Scale bar, 5 cm.

represent, at least in part, the activities of blue-green algae, as seem probable based on comparisons with modern stromatolites¹⁸, including *Conophyton*¹⁹, they would substantiate suggestions that photosynthesis was an effective source of oxygen 3,500 Myr ago²⁰. They also imply that the origin of terrestrial life lies much further back in geological time than 3,500 Myr.

This report is based on work supported by the NSF under grant EAR78-01642.

Received 26 October 1979; accepted 8 January 1980.

- Hickman, A. H. & Lipple, S. L. *Explanatory Notes for Marble Bar 250,000 Sheet* (Geol. Surv. W. Aust., 1978).
- Lowe, D. R. *Rev. Earth planet. Sci.* (in the press).
- Maslov, V. P. *Probl. Paleont.* 2-3, 327-342 (1937).
- Cloud, P. E. Jr. & Semikhatov, M. A. *Am. J. Sci.* 267, 1017-1061 (1969).
- Donaldson, J. A. in *Stromatolites* (ed. Walter, M. R.) 523-534 (Elsevier, Amsterdam, 1976).
- Hoffman, P. in *Stromatolites* (ed. Walter, M. R.) 599-612 (Elsevier, Amsterdam, 1976).
- Neumann, A. C., Gebelein, C. D. & Scoffin, T. P. *J. sedim. Petrol.* 40, 274-297 (1970).
- Pidgeon, R. T. *Earth planet. Sci. Lett.* 37, 421-428 (1978).
- Sangster, D. E. & Brooks, W. A. *Nature* 270, 423 (1977).
- Oversby, V. M. *Geochim. cosmochim. Acta* 40, 817-829 (1976).
- Delaeter, J. R. & Blockley, J. G. *J. geol. Soc. Aust.* 19, 363-370 (1972).
- Henderson, J. B. *Can. J. Earth Sci.* 12, 1619-1630 (1975).
- Jolliffe, A. W. *Econ. Geol.* 50, 373-398 (1955).
- Macgregor, A. M. *Trans. geol. Soc. S. Afr.* 43, 9-15 (1941).
- Schopf, J. W., Oehler, D. Z., Horodyski, R. J. & Kvenvolden, K. A. *J. Paleont.* 45, 477-485 (1971).
- Mason, T. R. & Von Brunn, V. *Nature* 266, 47-49 (1977).
- Dunlop, J. S. R., Muir, M. D., Milne, V. A. & Groves, D. I. *Nature* 274, 676-678 (1978).
- Awramik, S. M. in *Chemical Evolution of the Early Precambrian* (ed. Ponnamperna, C.) 111-132 (Academic, New York, 1977).
- Walter, M. R. in *Stromatolites* (ed. Walter, M. R.) 489-498 (Elsevier, Amsterdam, 1976).
- Schidlowski, M., Eichmann, R. & Junge, C. E. *Precamb. Res.* 2, 1-69 (1975).

Stromatolites 3,400-3,500 Myr old from the North Pole area, Western Australia

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Stromatolites are the least controversial evidence of early life; they are organosedimentary structures resulting from the growth and metabolic activity of microorganisms¹. Before this report, however, the oldest well established occurrence was in the 2,900-3,000 Myr Pongola Supergroup of South Africa²; five or six additional occurrences are known from the later Archean³. The only proposed example from older rocks is of a possibly stromatolitic microfabric from 3,500 Myr cherts in South Africa⁴; as yet that interpretation has not been supported by the discovery of macroscopic stromatolites. Here we describe stromatolites 3,400-3,500-Myr old from the Pilbara Block of Western Australia. These are the oldest firmly established biogenic deposits now known from the geological record.

Only in rocks younger than 2,300 Myr, recording the second half of Earth history, is our understanding of the course of biological evolution closely constrained by palaeontological, geochemical and sedimentary evidence^{5,6}. Objects interpreted as microfossils have been reported from 3,300-3,500 Myr cherts from Australia⁷ and Africa^{8,9}, and 3,770 Myr quartzites from Greenland^{10,11}, but none has yet found general acceptance¹²⁻¹⁴. Sulphur and carbon isotope data have been interpreted as indicating that the origin of microbial sulphate-reduction occurred between 2,700 and 3,300 Myr ago¹⁵⁻¹⁷ and that photosynthesising microorganisms may have originated even earlier than the deposition of the oldest known sediments

(3,770 Myr)¹⁸, but as yet relevant data are few and at least in the example of the carbon isotopes are subject to differing interpretations¹⁹. Kerogen is abundant in some Archean sedimentary rocks²⁰, but the interpretation of this is complicated by the fact that carbonaceous substances can be produced abiogenically²¹, as well as by heterotrophic and autotrophic organisms.

Structures resembling stromatolites are abundant in a chert-barite unit in the dominantly ultramafic-mafic metavolcanic Warrawoona Group of the eastern Pilbara Block (Fig. 1). Many of these structures seem to have formed by abiological processes (such as slump folding and silica deposition in voids), and others, while possibly biogenic, are not distinctive enough to be interpreted unambiguously. Earlier studies recorded the presence of possible stromatolitic fabrics²², clasts possibly formed by desiccation of microbial mats (some having oncolite-like overgrowths)⁷ and possible stromatolites²³. The structure we describe here has a relatively complex morphology which provides more criteria for biogenicity.

The chert-barite unit at North Pole consists of bedded shallow water metasediments 40 m thick that persist for at least 30 km along strike^{24,25}. The maximum metamorphic grade is in the lower greenschist facies, and the rocks have been subjected to little strain (ref. 26, and B. James, personal communication). Four sediment types are present: (1) those interpreted as silicified detrital carbonate rocks, often containing relict grains of carbonate and small (up to 1 cm) silica pseudomorphs after gypsum; (2) stratiform barite, some of which can be shown to have replaced evaporative gypsum^{24,25}; (3) silicified mafic volcanoclastic rocks; and (4) those interpreted as primary siliceous sediments, now cherts. A model lead age of 3,420 Myr has been obtained from galena within remobilised barite at this locality (J. Richards, personal communication). The volcanic Duffer

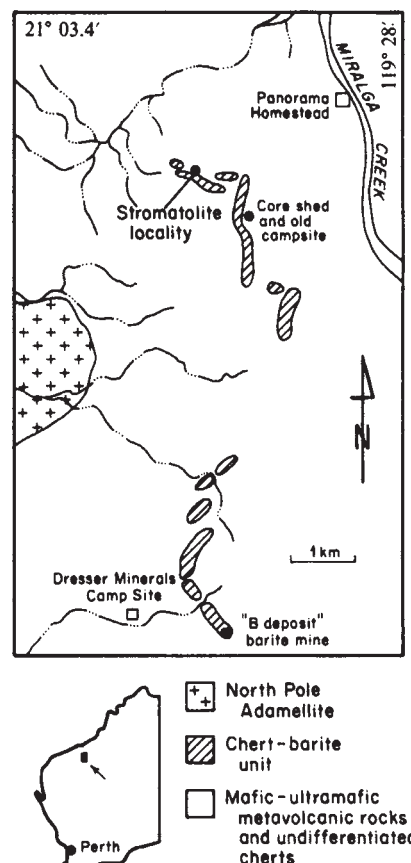


Fig. 1 Map of the northeastern part of the North Pole Dome, eastern Pilbara Block, showing the locality from which the stromatolite was collected. All rock units, apart from the post-tectonic intrusive North Pole Adamellite, are within the Warrawoona Group.

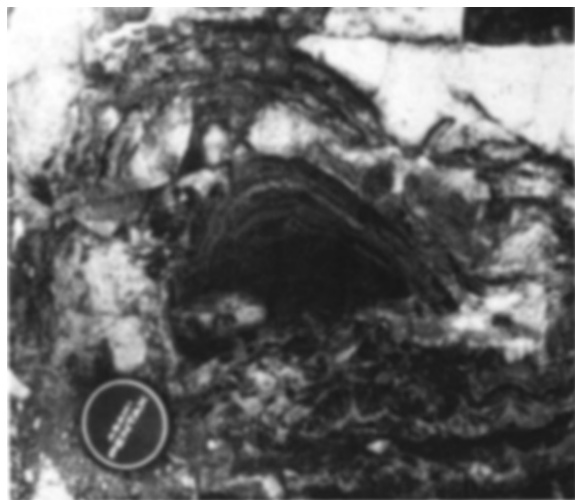


Fig. 2 Field photograph showing the exposed nodular and stratiform portions of the stromatolite. Scale, ~5 cm.

Formation, also within the Warrawoona Group and considered an approximate stratigraphic equivalent of the chert-barite unit²⁶, has yielded zircons that have been dated at $3,452 \pm 16$ Myr (ref. 27), and volcanics lower in the Warrawoona Group have recently been dated at $3,520 \pm 60$ Myr using Sm-Nd (P. J. Hamilton, personal communication).

The stromatolite described here is composed of dolomitic chert and was collected 5 m from the top of a 10.5-m thick exposure of bedded chert in an area of complex faulting 2.5 km WSW of Panorama Homestead (Fig. 1). Discussion of the possible original mineralic composition of the stromatolite is beyond the scope of the present paper. The bed underlying the stromatolite is composed of bands of clear to white chert, white agate, and red finely laminated jaspilite. The bands are separated by laminae of 1–2 mm wide rhombs of ankeritic dolomite. In outcrop, a single nodular structure 20-cm high by 25-cm wide (Fig. 2) was the only domical stromatolite visible; no similar structures were recognised in the same bed. The nodule protruded upward from a 20-cm thick bed of wavy laminated chert with lenses of intraclast and possibly ooid grainstone, into thin-bedded dolomitic chert. A specimen $60 \times 60 \times 40$ cm containing the nodule was collected and cut into slabs about 5-cm thick. This revealed several smaller, wavy laminated nodular stromatolites and a small escarpment encrusted by fine wavy laminae (Fig. 3).

It is apparent that the nodular and wavy laminated forms were primary sedimentary structures with relief, because undeformed overlying and adjacent beds wedge out against, abut against, or thin over the structures, and an erosional scarp has been encrusted by subsequently deposited laminae. The intraclasts found alongside the nodular structures are of two types: the first is thin (3–5 mm), flat, and similar in microstructure to the thin-bedded unlaminated chert interbedded with the stromatolites; the second is thin (~1 mm) and arcuate to undulose, and indistinguishable in microstructure from the stromatolite laminae. These latter delicate clasts probably could not have survived long transportation, and probably were derived locally from the accreting stromatolite subsequent to desiccation.

The stromatolites have a banded microstructure²⁸ with wavy and wrinkled laminae predominantly 50–200 μ m thick, although many laminae are as thin as 20 μ m (Fig. 4). Domical laminar structures up to 3 cm wide occur frequently within the stromatolites (Fig. 3), and many persist vertically for more than a centimetre, producing pseudocolumnar structures. Many of the domical structures are flat-topped, with sharply deflexed margins. Junctions between contiguous domes are V-shaped. Small, discrete columns occur rarely within the large stromatolite nodule. These laminar structures are closely comparable with those of younger stromatolites²⁹.

There are abiogenic structures that resemble stromatolites²⁹, but of all such structures, only one class seems to provide a plausible alternative to the biogenic interpretation of the North Pole structures. That is the possibility that they may be deposits from splashing water, such as the coniatolites described from the Persian Gulf³⁰. However, such an interpretation is not consistent with the abundant occurrence of intraclasts having the same microstructure as the stromatolite (a feature absent from coniatolites because they are not susceptible to desiccation due to their compact, hard nature), or with the absence of dripstone structures, abundant coated grains, and microreticulate ridges on lamina surfaces, features characteristic of coniatolites³⁰. We conclude that the structures are correctly identified as stromatolites. Research on the recognition and distribution of stromatolites in the Warrawoona Group and on the palaeoenvironmental setting, is continuing (R.B. and J.S.R.D.).

It seems that there was a benthic microbiota 3,400–3,500 Myr ago, but its biological affinities are unknown. The common opinion in the literature is that the presence of stromatolites establishes the former presence of cyanobacteria, but that is an unwarranted interpretation, especially for Archean stromatolites^{3,31}. There are living examples of bacterial stromatolites built by other than cyanobacteria, for example, by *Chloroflexus*, a green, photosynthetic, filamentous bacterium which presently constructs stromatolites in hot springs^{31–33}. Furthermore, it is reasonable to suggest that there was a time, before the advent of the appropriate cyanobacteria, when the dominant stromatolite-builders were organisms such as *Chloroflexus*. This is significant because among the bacteria only the cyanobacteria release oxygen during photosynthesis; so at

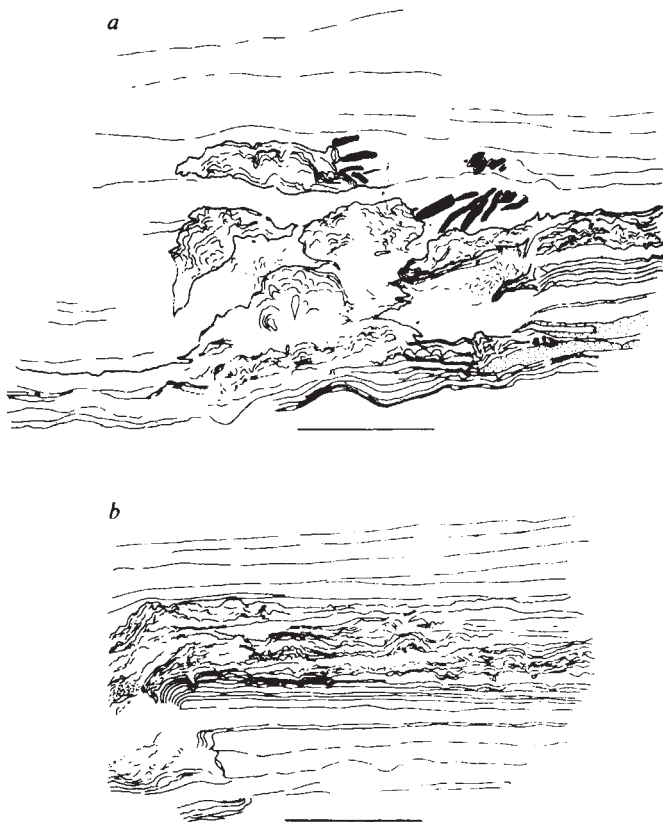


Fig. 3 Tracings of laminae revealed on sawn slab faces 2ii (parallel to and 13 cm further into the rock from the plane shown in Fig. 2) and A (at 90° to plane of a). The laminae consist of dolomitic chert. Note the three domical structures in the centre of a and the encrusted scarp to the lower left and the pseudocolumnar structures to the central left of b. The stipples represent possible ooid grainstones, the small dashes are intraclasts derived from the stromatolite and the large black tabular bodies are intraclasts derived from the overlying clear to white thinly bedded dolomitic chert. Scale bars, 10 cm.



Fig. 4 Photomicrograph in transmitted plain light of fine (20–100 μm) stromatolitic laminae composed of ferruginous dolomitic chert. Scale bar, 5 mm.

least for these very ancient rocks it is not possible to equate the presence of stromatolites with the former presence of oxygen-releasing photosynthesizers.

No microfossils have been found within the stromatolite, but a consideration of the microstructure allows us to draw some inferences about the morphology of the constructing organisms. As many laminae are as thin as 20 μm , these organisms must have been no larger than that, at least in one dimension. The regular, even, lamination of the stromatolite is comparable with that demonstrably produced by filamentous organisms in other stromatolites, and unlike that produced by coccoid organisms³⁴.

The microbiota seems to have inhabited an environment where it was intermittently exposed to the atmosphere and so inevitably was exposed to UV radiation, severe fluctuations in water potential³⁵, and diurnal temperature changes. Its presence may indicate that a protective ozone screen already existed. The microbiota had developed some strategy for coping with the danger of burial by sediment, which led to the accretion of the stromatolite, but it is not yet possible to say whether the strategy involved phototaxis, as in many younger stromatolites. Already at this very early stage in Earth history, a community of organisms existed that was adapted to life in an adverse, fluctuating environment.

The stromatolite described here was found by J.S.R.D. in 1977 during research for a doctoral project on the North Pole barite deposits. We thank the Bureau of Mineral Resources Geology and Geophysics, Australia, and the Geological Survey of Western Australia, for providing support in the field, and NASA Grant NSG 7489 and the NSF Waterman Foundation Award to J. W. Schopf for financial support. M.R.W. is on leave from the Bureau of Mineral Resources, Geology and Geophysics. J. M. Hayes, H. J. Hofmann, D. Gregg and L. Bettenay assisted with the collection of the specimen on which this study is based. We thank D. I. Groves for supervision and support, and J. W. Schopf, J. M. Hayes and M. Schidlowski for helpful comments.

Received 7 December 1979; accepted 20 February 1980.

- Walter, M. R. in *Stromatolites* (ed. Walter, M. R.) 1–3 (Elsevier, Amsterdam, 1976).
- Mason, T.R. & Von Brunn, V. *Nature* **266**, 47–49 (1977).
- Walter, M. R. in *Archaeal Cherty Metasediments: Their Sedimentology, Micropalaeontology, Biogeochemistry, and Significance to Mineralization* (eds Glover, J. E. & Groves, D. I.) 1–10 (Publications of the Geology Department and Extension Service, University of Western Australia, 2, 1978).

- Muir, M. D. & Grant, P. R. in *The Early History of the Earth* (ed. Windley, B. F.) 595–604 (Wiley, New York, 1976).
- Cloud, P. E. *Geol. Soc. S. Afr. Annex. Vol.* 79 (1976).
- Schopf, J. W. *Endeavour* **34**, 51–58 (1975).
- Dunlop, J. S. R., Muir, M. D., Milne, V. A. & Groves, D. I. *Nature* **274**, 676–678 (1978).
- Knoll, A. H. & Barghoorn, E. S. *Science* **198**, 396–398 (1977).
- Muir, M. D., Grant, P. R., Bliss, G. M., Diver, W. L. & Hall, D. O. in *Chemical Evolution of the Early Precambrian* (ed. Ponnampuram, C.) 155–170 (Academic, New York, 1977).
- Pflug, H. D. *Naturwissenschaften* **65**, 611–615 (1978); *Oberhessische Naturw. Z.* **44**, 131–145 (1978).
- Pflug, H. D. & Jaeschke-Boyer, H. *Nature* **280**, 483–486 (1979).
- Nagy, B., Zumberge, J. E. & Nagy, L. A. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1206–1209 (1975).
- Schopf, J. W. *Origins of Life* **7**, 19–36 (1976).
- Cloud, P. E. & Morrison, K. *Precamb. Res.* **9**, 81–91 (1979).
- Lambert, I. B., Donnelly, T. H., Dunlop, J. S. R. & Groves, D. I. *Nature* **276**, 808–811 (1978).
- Monster, J., et al. *Geochim. cosmochim. Acta* **43**, 405–413 (1979).
- Schidlowski, M. *Origins of Life* **9**, 299–311 (1979).
- Schidlowski, M., Appel, P. W. U., Eichmann, R. & Junge, C. E. *Geochim. cosmochim. Acta* **43**, 189–199 (1979).
- Perry, E. C. & Ahmad, S. N. *Earth planet. Sci. Lett.* **36**, 280–284 (1977).
- Reimer, T. O., Barghoorn, E. S. & Margulis, L. *Precamb. Res.* **9**, 93–104 (1979).
- Miller, S. L. & Orgel, L. E. *The Origins of Life on Earth* (Prentice Hall, Englewood Cliffs, 1974).
- Dunlop, J. S. R. thesis, Univ. Western Australia (1976).
- Dunlop, J. S. R. & Groves, D. I. *Geol. Soc. Am. Abstr. Prog.* **10**, 393.
- Barley, M. E., Dunlop, J. S. R., Glover, J. E. & Groves, D. I. *Earth planet. Sci. Lett.* **43**, 74–84 (1979).
- Dunlop, J. S. R. in *Archaeal Cherty Metasediments: Their Sedimentology, Micropalaeontology, Biogeochemistry, and Significance to Mineralization* (eds Glover, J. E. & Groves, D. I.) 30–38 (Publications of the Geology Department and Extension Service, University of Western Australia, 2, 1978).
- Hickman, A. H. & Lipple, S. L. *Explanatory Notes for Marble Bar 1:250 000 Sheet* (Geol. Surv. W. Austral., 1978).
- Pidgeon, R. T. *Earth planet. Sci. Lett.* **37**, 421–428 (1978).
- Walter, M. R. in *Stromatolites* (ed. Walter, M. R.) 687–692 (Elsevier, Amsterdam, 1976).
- Walter, M. R. (ed.) *Stromatolites* (Elsevier, Amsterdam, 1976).
- Purser, B. H. & Loreau, J. P. in *The Persian Gulf* (ed. Purser, B. H.) 343–376 (Springer, Berlin, 1973).
- Walter, M. R., Bauld, J. & Brock, T. D. *Science* **178**, 402–405 (1972).
- Doemel, W. N. & Brock, T. D. *Science* **184**, 1083–1085 (1974); *Appl. envir. Microbiol.* **34**, 433–452 (1977).
- Walter, M. R. in *Stromatolites* (ed. Walter, M. R.) 489–498 (Elsevier, Amsterdam, 1976).
- Monty, C. L. V. in *Stromatolites* (ed. Walter, M. R.) 193–249 (Elsevier, Amsterdam, 1976).
- Brock, T. D. in *Stromatolites* (ed. Walter, M. R.) 141–148 (Elsevier, Amsterdam, 1976).

How do fish break the speed limit?

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Many studies have shown that tail beat frequency of teleost fish is closely related to their swimming speed. These findings were developed further by Wardle¹, who showed that maximum tail beat frequency and thus swimming speed could be predicted by measuring the twitch contraction time of the fast swimming muscles. The contraction time increases with the length of the fish and decreases with increase of temperature. Several authors have observed and recorded swimming speeds greater than the predicted speed limit. For example, Stevens and Neil² report that skipjack tuna (*Katsuwonus pelamis* (L), length (L) 0.5 m) could swim at a speed of 10 m s⁻¹ at 32 °C, whereas the maximum predicted speed (extrapolating Wardle's¹ data) was only 8 m s⁻¹. Walters and Fiersteine³ recorded a yellowfin tuna (*Thunnus albacores* (Bonnatare), L = 0.98 m) moving at 20.8 m s⁻¹ and Wahoo (*Acanthocybium solandri* (Cuvier), L = 1.13 m) at 21.4 m s⁻¹. These two records show just twice the predicted maximum swimming speeds. Unfortunately no observations of the tail beat frequency were made during these high-speed swim records. Brill and Dizon⁴ have shown that five skipjack tuna (L 0.37–0.43 m) had muscle contraction times similar to non-scombroid teleost species of this size measured by Wardle¹. It seems therefore that the tuna family (Scombridae) do not have faster maximum tail beat frequency than other teleosts of the same size and temperature. We show here how this paradox can be solved by the fish using a different swimming style, requiring greater power and efficient interaction between the propelling surfaces and the water, but allowing the fish to move twice as far for each tail beat.

The normal relationship between tail beat frequency and speed can be explained by a simple analysis of the fish's swimming movements. We have analysed² the lateral body movements of cod, *Gadus morhua*, swimming at constant speeds and defined a two-wave system which allowed exact description of swimming movements in most teleost fish. Fish move forwards by means of a series of waves of contraction of the lateral muscles on alternate sides, starting in the myotomes behind the head and ending where the last myotome inserts on the tail. A wave causes lateral displacement of body and fins and these in turn cause forward motion by reaction with the water. During constant speed swimming the speed (v) of the wave moving from head to tail is always slightly greater than the forward swimming speed (u); so that u/v is less than 1. The wave on the body is S-shaped when seen from above and has a measurable wavelength λ_b . The amplitude of the wave is small in the head region and greatest at the tail tip where it is normally 0.2 times body length. The track traced by the tail tip through the water forms a second wave with wavelength λ_s , which is always shorter during constant speed swimming than λ_b , but has the same period (T); λ_s can be much longer or much shorter than λ_b , depending on whether the fish is slowing down or accelerating. It is easy to measure λ_s but measurement of λ_b and its velocity (v) requires accurate identity and location of the wave crests on images of the swimming fish. Towards the head, the amplitude of the wave can be very small and the wave crests difficult to identify. New techniques for filming and accurate analysis enabled us to measure the wave parameters and development of the relationship between the two wave systems discussed before⁵. We confirmed that $u/v = \lambda_s/\lambda_b$. Previous studies have shown that λ_s which is equal to the distance moved forwards after completion of one tail beat cycle or the passage of one wave, is near to 0.7 times body length (L). For all the species studied, including goldfish (*Carassius auratus*) and tuna, this figure holds, and tail beat frequency gives a straight line when plotted against swimming speed (measured in lengths per second) during constant speed swimming and over a wide range of speeds⁶⁻⁹.

With u/v always slightly less than 1, λ_s always close to 0.7 L and λ_b always longer than λ_s (in cod $\lambda_b = 0.85 L$, ref. 4) it becomes evident that there is relatively little variation in the relation between λ_b and L . All those species where tail beat frequency has been related to constant swimming speeds have therefore shown slightly more than 1 λ_b within the straight body length (L).

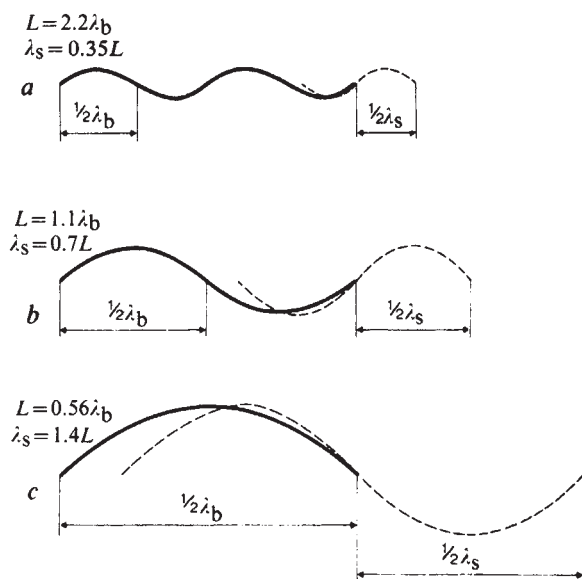


Fig. 1 Firm lines represent the wave (wavelength λ_b) on the body of a fish with straight length L . The dashed lines show the track of the tail tip (wavelength λ_s) after one completed tail beat. Note the distance moved forwards after two contractions of the lateral swimming muscles: a, 0.35 L ; b, 0.7 L ; c, 1.4 L .

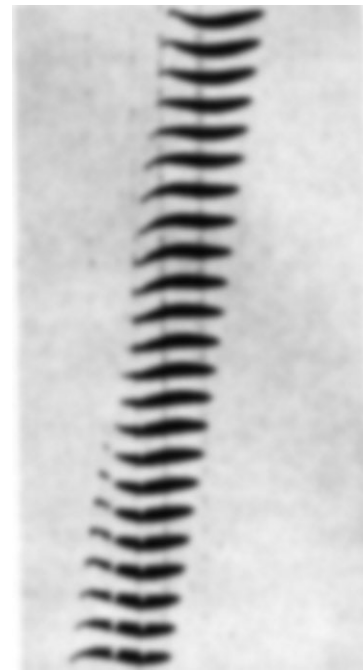


Fig. 2 Mackerel *Scomber scombrus* L., length 0.345 m, speed 2.9 m s^{-1} , swimming in the style shown in Fig. 1b, printed from 16-mm cine film made at $200 \text{ frames s}^{-1}$ (Locam camera). Fish were illuminated with 150-W tungsten lamp, the light source guided by fibre optics to shine from points beside the camera lens to create silhouettes of the fish moving across a marked screen of reflex reflector material (3M high intensity Scotchlite). The reference lines are 25 cm apart. The tail sweeps from left to right in 35–40 ms, a time close to the twitch contraction time at 12°C .

To develop this important point let us compare the normal fish discussed so far with one fish showing two complete waves on its body so that $L \approx 2.2 \lambda_b$ and another fish of the same length but with only half a wavelength on its body so that $L \approx 0.56 \lambda_b$ (Fig. 1). Consider for each case the result of completing one tail beat cycle during constant speed swimming in time T . The track of the tail tip in each case is indicated by the dashed lines, and the distance moved forwards is a different λ_s in each case. In Fig. 1a the fish moves forwards less than 0.35 L . In the normal fish represented by Fig. 1b the forward movement is 0.7 L . In Fig. 1c, showing the fish with $L \approx \frac{1}{2} \lambda_b$, the distance covered is about 1.4 L . That is exactly twice the progress of the normal fish in the same time T . In all three cases the distance moved is the result of one contraction of the right and one contraction of the left lateral swimming muscles. This means that the shortest time in which each of the illustrated manoeuvres (Figs 1a–c) could be completed is twice the twitch contraction time as discussed by Wardle¹. This argument suggests that if L could ever approach or even equal $\frac{1}{2} \lambda_b$, the fish could bound forwards at up to twice the speed of the normal fish using the same contraction time. However, consider the problems of swimming at maximum speed with $L = \frac{1}{2} \lambda_b$. It is reasonable to suppose that normal fish swimming at maximum speed are contracting nearly the whole of the fast, white anaerobic muscle in each myotome in order to develop sufficient force in the wave to counteract the drag of the water on the fast moving body. This force (F) increases with the swimming speed to the power 1.8 where $F = \frac{1}{2} \rho A u^2 1.2 C_f$ (ref. 10). The flow around fish longer than 0.50 m swimming at 5 m s^{-1} at 30°C will be turbulent and C_f , the drag coefficient, will be proportional to $\text{Re}^{-0.2}$ and so proportional to $u^{-0.2}$. F is then proportional to $u^{1.8}$. (Re is Reynolds number, ρ is the density of water and A the body surface area.) The power (Fu) increases with $u^{2.8}$. That means to double the speed, seven times the power is required; equivalent to seven times the volume of swimming muscle. A fish swimming with $\frac{1}{2} \lambda_b = L$ might reason-

ably be expected to have a body diameter three to six times greater than a fish of the same actual length but with $L = \lambda_b$. However, a greater length of each lateral muscle will be involved in developing the movement when $L = \frac{1}{2}\lambda_b$, so this could be an overestimate. The increased muscle power will require extra strengthening of the spinal column and the surfaces which propel the fish must also be able to shed effectively seven times the power. Power consumption has been measured in cod (length 0.73 m) swimming at 3 m s^{-1} at about 30 W kg^{-1} , and an estimated 100 W kg^{-1} was required to swim at its maximum speed of 5.5 m s^{-1} (ref. 10). A cod-like fish swimming at twice this speed might need to dissipate up to 700 W kg^{-1} . The thick body, strong spinal column and stiff high tail blade of the Scombridae does satisfy to some degree these points.

Is there real evidence that fish can swim with a body wavelength longer than the body length? A graph by Magnuson, summarising the relationship between tail beat frequency and swimming speed in the Scombroidei (fig. 16 in ref. 11), shows a series of measurements where at slower swimming speeds the distance moved forwards, λ_s , for completion of one tail beat is greater than one fish length (L). However, as the speed increases λ_s becomes less than $1L$ and all the other scombrid data presented in the figure indicate λ_s similar to other teleosts at

about $0.7L$. It is possible that where λ_s was found to be greater than $1L$, the fish were slowing down. Preliminary measurements of films we have made show that the mackerel *Scomber scombrus* L. (Scombroidei) (lengths 0.33 and 0.345 m) swim with a complete 'S' wave on the body (Fig. 2) at 1.17 and 2.9 m s^{-1} with $u/v = 0.86$, $\lambda_s = 0.76L$ and $\lambda_b = 0.89L$, all parameters similar to those for the cod. The cine film frames (Fig. 2) show the larger fish swimming at 2.9 m s^{-1} , using the swimming muscles close to their twitch shortening time (40 ms, 9°C) with a tail beat frequency of 11 Hz. The observed high speed swimming of some fish species does exceed the speed limit set by current knowledge of fish swimming, which can only be explained if these fish either use a style of swimming where $\frac{1}{2}\lambda_b = L$, or if they have muscle with much shorter contraction time than usual. To establish the truth there is an urgent need to measure the muscle contraction times, particularly of those species and sizes for which claims to high speed have been made, and it is important that future recordings of high speed swimming should aim to include among other evidence a tail beat frequency record.

We are grateful for a Royal Society Fellowship in the European Science Exchange Programme (to J.J.V.) and NATO grant No 1127.

Received 7 December 1979; accepted 13 February 1980.

1. Wardle, C. S. *Nature* **255**, 725–727 (1975).
2. Stevens, E. D. & Neil, W. M. in *Fish Physiology* **7** (eds Hoar, W. S. & Randall, D. J.) 316–361 (Academic, New York, 1978).
3. Walters, V. & Fiersteine, H. L. *Nature* **202**, 208–209 (1964).
4. Brill, R. W. & Dizon, A. E. *Environ. Biol. Fish.* **4**, No 3 (1979).
5. Videler, J. J. & Wardle, C. S. *Netherlands J. Zool.* **28**, 465–484 (1978).
6. Bainbridge, R. J. *exp. Biol.* **35**, 109–133 (1958).
7. Hunter, J. R. & Zweifel, J. R. *Fish Wild. Serv. Fish Bull.* **69**, 253–266 (1971).
8. Pyatetskii, V. Y. in *Hydrodynamic Problems of Bionics*, *Bionika* No 4 (1970) translated from Russian JPRS 52605, 12–23. (National Technical Information Service, Washington DC 1971).
9. Hudson, R. C. L. *J. exp. Biol.* **58**, 509–522 (1973).
10. Wardle, C. S. & Reid, A. in *Fisheries Mathematics* (ed. Steele, J. H.) 171–191 (Academic, New York 1977).
11. Magnuson, J. J. in *Fish Physiology* **7** (eds Hoar, W. S. & Randall, D. J.) 240–316 (Academic, New York, 1978).

Evolution of the orang-utan

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The common assumption that the lineage leading to modern orang-utans has always been arboreal is based on general principles of irreversibility and parsimony rather than on any direct evidence of postcranial morphology in ancestral species. We argue here that the available evidence, albeit indirect and circumstantial, justifies the concept of a Pliocene orang-utan ancestor at least as terrestrial as the modern chimpanzee.

The fossil record of undoubted orang-utans consists solely of Pleistocene age teeth, found in sites widespread throughout South-East Asia, including the Chinese provinces of Yunnan, Kwangsi, Kwangtung and Kweichow^{1–3} and North Viet Nam (Simonetta, cited in ref. 4). The association of orang specimens at these localities with a Middle Pleistocene *Stegodon-Ailuropoda* fauna contributes little to an understanding of orang-utan evolution, because the species comprising such faunas give evidence of tropical, subtropical and temperate adaptations^{5–8}. If further study of specimens held in mainland China becomes possible, a preliminary report of fossil orang-utans at a site in Shantung Province⁹ could prove significant. This locality is more than 10° in latitude further north than the well-verified south-eastern fossil orang sites, in a region that was partial desert during maximum glacial advances and open grassland during interglacials^{10,11}.

Subfossil orang-utans, dated at 30,000–40,000 BP, have been found on Sumatra and Borneo. The maxillary occlusal surface area has been estimated to be 12.9% larger in the Sumatran subfossil, *P. pygmaeus paleosumatensis*, than in extant *Pongo*¹².

The Chinese Middle Pleistocene subspecies, *P. p. weidenreichi*, has teeth even larger than those of the Sumatran subfossil^{12,13}. The tooth size of these fossil and subfossil species indicates that either these animals were larger bodied than the living species, with a tooth size to body size ratio that could have been similar to that of extant orangs, or that they were roughly the same body size as modern *Pongo*, but with larger teeth. Both of these conditions imply the existence of a more terrestrial species. The adult male orang-utan is already the largest arboreal animal known, and field observations suggest that larger individuals would have difficulty supporting their weight in trees. If the fossil species was larger than the modern one, adult males, at least, would have been mostly terrestrial.

If, on the other hand, they were animals with larger teeth relative to body size than living orang-utans (who have relatively large teeth among the extant great apes^{14,15}), we are aware of two plausible explanations. First, the large teeth could be a dietary adaptation. This would not be particularly significant evidence for either an arboreal or a terrestrial niche¹⁶. Alternatively, the relatively large teeth could be a consequence of rapid phyletic dwarfing in body size. The Middle Pleistocene orang-utan is a mainland species, while the subfossils and extant species are restricted to islands. It is unusual for mainland and island populations of a lineage to remain the same size¹⁷, and during the Early and Middle Pleistocene a decrease in the body size of large herbivores on islands was common. When body size undergoes rapid reduction, tooth size decreases more slowly^{18,19}, resulting in relative megadonty.

Thus, subfossil orang-utans were probably arboreal (and therefore little if any larger in body size than living orang-utans) for it seems unlikely that 40,000 yr has been sufficient for the modern arboreal specialisations to evolve. Perhaps arboreality developed from semi-terrestrial, semi-arboreal populations on the islands in response to human predatory pressures²⁰, which accelerated body size reductions. The still larger-toothed, larger-bodied Middle Pleistocene mainland orang-utans would have been less arboreal, and more chimpanzee-like in their use of terrestrial habitats.

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Additional evidence for our hypothesis comes from an examination of morphology and behaviour of the living species. In social structure and craniofacial morphology, the orang-utan is an odd animal. The sex difference in the body size of orang-utans is among the greatest of all primates^{21,22}. This is usually explained on the basis of aggressive competition between adult males^{20,21,23-27}, even though sexual selection is not clearly indicated by field observations. For example, Horr²⁴ admits that "direct evidence for male competition in the form of dominance or aggressive encounters is limited" and Leutenegger and Kelley²¹ observe that their acceptance of intrasexual selection is "partly based on negative evidence for other selective factors". While allometry²⁸, bioenergetic and reproductive constraints on female size²⁹ or a maximisation of food resources by niche separation due to body size²² remain possible factors, it is also plausible that the sex difference and large body size of orang-utans are remnants ('heritage' characters) of a more terrestrial pattern³⁰ (accompanied perhaps by a different type of social structure, of which we can know nothing).

Several aspects of dental and craniofacial morphology differ from those seen in most primate arboreal herbivores. Orang-utans have high crowned, low cusped (bunodont) cheek teeth with marked occlusal wrinkling, different from those of other catarrhine primates which exhibit well-defined cusps and shearing facets^{31,32}. The enamel on the occlusal surfaces of their cheek teeth is relatively and absolutely thicker than in any other species of living primate yet examined³³. Enamel thickness is relatively thicker on the occlusal surfaces of species of *Macaca*, *Papio*, *Theropithecus* and *Cercopithecus* (*C. aethiops*) than of species of *Colobus*, *Presbytis*, *Alouatta*, *Ateles*, *Pan* or *Gorilla*³⁴. This appears to separate species with potentially more abrasive (omnivorous) diets from those with less abrasive (folivorous, frugivorous) diets. *Pongo* is the apparent exception^{33,34}.

The mandibular condyle of the orang-utan is high above the occlusal plane and the ramus forms a relatively acute angle with the mandibular corpus. The dental arches are relatively forward in position and tilted up³⁵. Although Biegert³⁵ suggests that these changes parallel those seen in *Alouatta* and can be explained by hyolaryngeal specialisations, Hershkovitz³⁶ argues that *Alouatta* craniofacial morphology has nothing to do with hyoid hypertrophy, but rather with masticatory adaptations to herbivorous browsing, and Zingesser³⁷ confirms that several of these features are characteristic of folivory in New World monkeys.

Living orang-utans therefore have unusual (for an arboreal frugivore) dental and craniofacial adaptations. This suggests either that we do not yet have a clear understanding of the relationships between diet and anatomy in living arboreal or frugivorous primates (which is a distinct possibility), or that ancestral orangs were not arboreal frugivores. What they were we cannot say, but that they have retained a number of characteristics from ancestors adapted to doing very different things seems a real possibility.

Consideration of a terrestrial phase in orang-utan evolution goes hand-in-hand with a growing awareness within the past 5 yr that Miocene hominoid evolution is much more complex than envisioned 15 yr ago³⁸. Typical Neogene hominoids (including our hypothetical Pliocene orang-utan) were probably neither forest nor savannah dwellers, but woodland creatures. Most of these species did not resemble living hominoids. Whether some of them are ancestral to living hominoids, or whether they all became extinct without issue, it seems to us that an even wider range of possible evolutionary scenarios must be considered by students of hominoid evolution than was the case a few years ago. Selecting the correct one is probably not possible given the still poor state of the hominoid fossil record. It will not be made easier unless we realise that past hominoid morphologies, distribution and habitats were probably rather different from those of today; that we should not cling too tenaciously to principles of irreversibility or parsimony; that parallelism in hominoid evolution may have been much more widespread than some of

us have thought; and that we should intentionally begin searching for more heterodox explanations.

For comments on early drafts we thank Peter Andrews, Matt Cartmill, John Fleagle, Edward Harris, Michael Rose, Jack Stern, Russell Tuttle, Rimas Vaisnys and Sherwood Washburn. R.J.S. was supported by National Research Service Award DE 05134 (NIDR).

Received 9 November 1979; accepted 1 February 1980.

1. Kahlke, H. D. *Vertebr. palasiat.* **2**, 104-108 (1961).
2. Kahlke, H. D. *Asian Perspect.* **15**, 5-14 (1972).
3. Delson, E. *Paleoanthropology in the People's Republic of China* (eds Howells, W. W. & Tsuchitani, P. J.) 40-65 (National Academy of Sciences, Washington DC, 1977).
4. Groves, C. P. *Mammalian Species* No. 4 (American Society of Mammalogy, 1971).
5. von Koenigswald, G. H. R. *Anthrop. Pap. Am. Mus. nat. Hist.* **43**, 291-326 (1952).
6. Simons, E. L. & Eitel, P. *Scient. Am.* **221**(1), 76-85 (1970).
7. Pilbeam, D. R. *Nature* **225**, 516-519 (1970).
8. Pilbeam, D. R. *Current Arguments on Early Man* (ed. Konigsson, L.-K.) (Pergamon, Oxford, in the press).
9. Aigner, J. S. *Anthropologie, Brno* **10** (Nos. 2 & 3), 39-50 (1972).
10. Frenzel, B. *Science* **161**, 637-649 (1968).
11. Hsu, J. *Scientia sin.* **15**, 410-414 (1966).
12. Hooijer, D. A. *Zool. Meded. (Mus. Leiden)* **29**, 175-301 (1948).
13. Hooijer, D. A. *Evolution* **3**, 125-128 (1949).
14. Pilbeam, D. R. & Gould, S. J. *Science* **186**, 892-901 (1974).
15. Gingerich, P. D. *Am. J. phys. Anthropol.* **47**, 395-398 (1977).
16. Kinzey, W. G. *J. hum. Evol.* **3**, 193-203 (1974).
17. Bonner, J. T. *Paleont. Soc. Mem.* **2**, 1-15 (1968).
18. Gould, S. J. *Am. Zool.* **15**, 351-362 (1975).
19. Marshall, L. G. & Corruccini, R. S. *Paleobiology* **4**, 101-119 (1978).
20. MacKinnon, J. *Anim. Behav.* **22**, 3-74 (1974).
21. Leutenegger, W. & Kelley, J. T. *Primates* **18**, 117-136 (1977).
22. Clutton-Brock, T. H., Harvey, P. H. & Rudder, B. *Nature* **269**, 797-800 (1977).
23. Rodman, P. S. *Comparative Ecology and Behavior of Primates* (eds Michael, R. P. & Crook, J. H.) 171-209 (Academic, London, 1973).
24. Horr, D. A. *Primate Behavior* (ed. Rosenblum, L. A.) **4**, 307-323 (Academic, New York, 1975).
25. Galdikas-Brindamour, B. *Nat. geogr. Mag.* **148**, 444-473 (1975).
26. MacKinnon, J. *Primates* **18**, 747-772 (1977).
27. Rijksen, H. D. *Fifth int. Cong. Primatol.* 373-379 (Karger, Basel, 1975).
28. Leutenegger, W. *Nature* **272**, 610-611 (1978).
29. Ralls, K. *Am. Nat.* **111**, 917-938 (1977).
30. MacKinnon, J. *Oryx* **11**, 141-191 (1971).
31. Kay, R. F. *Am. J. phys. Anthropol.* **43**, 195-216 (1975).
32. Walker, P. & Murray, P. *Primate Functional Morphology and Evolution* (ed. Tuttle, R. H.) 135-150 (Aldine, Chicago, 1975).
33. Molnar, S. & Gantt, D. G. *Am. J. phys. Anthropol.* **46**, 447-454 (1977).
34. Gantt, D. G. thesis, Washington Univ. (1977).
35. Biegert, J. *Classification and Human Evolution* (ed. Washburn, S.) 116-145 (Aldine, Chicago, 1963).
36. Hershkovitz, P. *Folia primatol.* **13**, 213-240 (1970).
37. Zingesser, M. R. *Folia primatol.* **20**, 351-390 (1973).
38. Simons, E. L. & Pilbeam, D. R. *Folia primatol.* **3**, 81-152 (1965).

Dispersal and the sex ratio

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It has been shown by Fisher that a 1:1 sex ratio should be evolutionarily stable as there would otherwise be a frequency-dependent advantage to the rarer sex¹. Hamilton pointed out that Fisher's argument depends on the assumption of population-wide random mating, and showed that a female-biased sex ratio was expected in a model in which mating occurred within small local subgroups before population-wide dispersal of mated females. We consider here the sex ratio under some other models of dispersal in a geographically structured population.

For simplicity we consider one-dimensional models, and we suppose that the habitat consists of $2M+1$ discrete patches placed round a circle and labelled $-M, \dots, -1, 0, 1, \dots, M$. In our first model we suppose that there is one mated pair in each patch, which produces k offspring and then dies. Male and female offspring disperse a distance j with probabilities p_j and q_j , respectively ($-M \leq j \leq M$). After dispersal, one pair establishes itself in each patch in the next generation, the male and female partners being chosen at random from the individuals in the patch.

To find the sex ratio which is evolutionarily stable, suppose that the pair in patch 0 has sex ratio s (producing sk sons and $(1-s)k$ daughters), while all other pairs have sex ratio r . To see whether the pair in patch 0 is at a selective advantage to other pairs, let the genes of this pair at a typical autosomal locus be coloured red. We shall now calculate then expected number of red genes in mated pairs in the next generation.

After dispersal there are $r(1-p_i)k$ males with no red genes and $sp_i k$ with two red genes in patch j , with corresponding expressions for females. The expected number of red genes in the successful pair in patch j is

$$\alpha_j = \frac{2sp_j}{sp_j + r(1-p_j)} + \frac{2(1-s)q_j}{(1-s)q_j + (1-r)(1-q_j)} \quad (1)$$

The total expected number of red genes in successful pairs in the next generation is $\alpha = \sum \alpha_j$. Writing $\varepsilon = (s-r)$ and then expanding α in a Taylor series we find that

$$\alpha = 4 + \varepsilon \left\{ \frac{2}{r} (1 - \sum p_i^2) - \frac{2}{(1-r)} (1 - \sum q_i^2) \right\} \quad (2)$$

to order ε . The evolutionarily stable sex ratio is the value of r which makes the coefficient of ε zero, given by

$$\frac{(1-r)}{r} = \frac{1 - \sum q_i^2}{1 - \sum p_i^2} \quad (3)$$

[The above argument is only approximate as it does not track the frequency of a gene determining sex ratio from one generation to the next and since we have only sought the value of r which is at a selective advantage over any mutant sex ratio s present in a single patch. We believe that the result is qualitatively correct, but a more exact analytical treatment of this problem would be valuable.]

Equation (3) can be interpreted as meaning that the evolutionarily stable sex ratio is biased in favour of the sex which disperses more widely and/or more evenly. The mechanism underlying this effect is the competition between siblings of the same sex which has been built into the model; siblings of the sex which disperses further (and/or more evenly) are less likely to be in the same patch after dispersal than siblings of the other sex. The equilibrium sex ratio in equation (3) is the point at which the advantage of producing offspring of the sex with less sibling competition is balanced by the disadvantage of producing offspring of the commoner sex. This mechanism also accounts for the female-biased sex ratio in Hamilton's model^{2,3}, as in this case there is competition between brothers for mates but no competition between sisters.

The above model assumes that mating occurs after dispersal (in contrast with Hamilton's model) and that individuals disperse independently of each other. The second assumption is more likely to be satisfied in animals dispersed passively by external physical forces (for example, marine plankton) than in animals which can control their own movement and can therefore mitigate the effect of competition for space by spacing themselves out. The assumption of independent, passive dispersal is also likely to be appropriate for plants; we shall now extend the model to the problem of resource allocation to male and female functions in hermaphrodite annual plants.

As before we suppose that the habitat consists of discrete patches round a circle. One plant grows in each patch, and each plant produces both pollen and seed. Pollen disperses a distance j with probability p_j , the seed in any patch is fertilised at random by the pollen arriving there, and then disperses a distance j with probability q_j . Of the seed arriving in a patch, exactly one is successful in establishing itself as a mature plant in the next generation.

We suppose that a plant can produce either N pollen grains or n ovules or any linear combination of rN pollen grains and $(1-r)n$ ovules ($0 \leq r \leq 1$). The parameter r is the proportion of its resources allocated to male as opposed to female functions, and is the analogue of the sex ratio in dioecious organisms. To find the evolutionarily stable value of r , suppose that a plant in a single patch has a sex ratio s while all other plants have a sex ratio r . Using the argument invoked above, we find that the evolutionarily stable sex ratio is given by

$$\frac{1-r}{r} = \frac{1+p_0 - \sum q_i^2 - \sum p_i q_{i-1} q_i}{1 - \sum p_i^2} \quad (4)$$

This is a rather complicated expression, but it is clearly possible to obtain either a male-biased or a female-biased sex ratio, depending on the relative dispersal distance of seed and pollen. In general, we may suppose that pollen dispersal has a mode at zero, so that $p_0 \geq p_j$. In this case

$$\sum p_i q_{i-1} q_i \leq p_0 \sum q_{i-1} q_i = p_0$$

so that

$$\frac{1-r}{r} \geq \frac{1 - \sum q_i^2}{1 - \sum p_i^2} \quad (5)$$

Comparing equations (3) and (5), we conclude that there is a tendency for the sex ratio to be biased towards male or female accordingly as pollen or seed disperses more, but that in addition there is some bias towards the female (seed production). This can be attributed to the fact that male (pollen) dispersal occurs in the gamete stage before fertilisation, while female (seed) dispersal occurs in the zygote stage after fertilisation.

The above argument places no restriction on selfing. If selfing is avoided, then effective pollen must come from a different plant. The argument goes through as before if we consider only effective pollen, and equation (4) remains valid if we replace p_i by the truncated distribution p'_i , defined by

$$p'_0 = 0 \\ p'_j = p_j / (1 - p_0), \quad j \neq 0$$

In general, the sex ratio will be slightly more male-biased with avoidance of selfing than if selfing is allowed.

We conclude that differential sibling competition is an important factor in determining the equilibrium sex ratio in a geographically structured population, and can lead to a bias in favour either of males or of females. It has been suggested⁴ that sibling mating is a factor which determines departures from a 1:1 sex ratio in this situation, but in our view⁵ sibling mating is only important as an indicator of the competition between brothers for mates. It is not possible to infer a general relationship between the amount of sibling mating or inbreeding and the sex ratio without considering the means by which inbreeding is caused.

Empirical evidence of the effect of sibling competition on the sex ratio has been found in the bushbaby *Galago crassicaudatus*⁶. There seems to be a male-biased sex ratio, which can be attributed to competition between female kin (sisters, and mothers and daughters) for local limiting resources of high quality food required by pregnant and nursing females.

This investigation was supported by a grant from the National Sciences and Engineering Research Council, Canada.

Received 5 November 1979; accepted 4 February 1980.

1. Fisher, R. A. *The Genetical Theory of Natural Selection* (Oxford University Press, 1930).
2. Hamilton, W. D. *Science* **156**, 477-488 (1967).
3. Taylor, P. D. & Bulmer, M. G. *J. theor. Biol.* (submitted).
4. Maynard Smith, J. *The Evolution of Sex* (Cambridge University Press, 1978).
5. Taylor, P. D. & Bulmer, M. G. *Am. Nat.* (submitted).
6. Clark, A. B. *Science* **201**, 163-165 (1978).

Measurement of gene flow in *Lupinus texensis*

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Gene flow, the movement or dispersal of genes within or among populations, is a crucial factor in determining the structure and cohesiveness of species and populations. Spatial restriction of gene flow can lead to non-random mating and to subdivision of a population into genetic neighbourhoods. On the other hand, extensive gene flow over large geographical areas can overshadow the influence of selection, leading to genetic similarity among populations and increased uniformity within a species. The extent and magnitude of actual gene flow are thus relevant to a broad range of disciplines. While the central role of gene flow is widely recognised, actual measurements of gene flow in either plants or animals are few. In plants gene flow can occur via seed or pollen dispersal. Levels of gene flow due to pollen dispersal have usually been inferred, either by marking pollen with a chemical or radioactive label for wind pollinated species or by determining the distribution of pollinator foraging flight distances for insect-pollinated species. Measurements of gene flow via pollen movement in plants have been limited to studies of a few agricultural species, where the minimum separation distance required to isolate stocks genetically was of interest^{1,2}. Here I report the measurement of the pollen component of gene flow in the insect-pollinated species, *Lupinus texensis*, compare the actual gene flow distribution with the distribution inferred from pollinator flight movements, and finally, determine the genetic neighbourhood size of this species from the pollen and seed dispersal distributions. Actual gene flow via pollen is found to be greater than would be inferred from pollinator movement alone. Gene flow and neighbourhood size are nevertheless very restricted.

Lupinus texensis Hook. (Leguminosae), the Texas bluebonnet, is a winter annual plant of widespread occurrence in central Texas. It is obligately outcrossing, and the pollen vectors are bees, predominantly *Bombus pennsylvanicus* and *Apis mellifera*. Seeds of *L. texensis* are dispersed over distances up to 4 m by the explosive dehiscence of the seed pod.

The pollen component of gene flow was studied in an experimental population of *L. texensis* using as genetic markers allozymes of the phosphoglucose isomerase-1 locus (*Pgi-1*). The *Pgi-1* locus is variable in natural *L. texensis* populations which often contain as many as five mendelian alleles. Flowering plants grown in a greenhouse from field-collected seeds were assayed

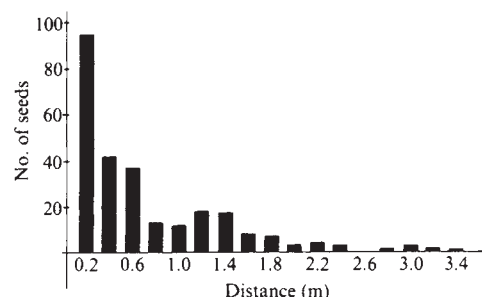


Fig. 2 Seed dispersal in *Lupinus texensis*. Seed dispersal is plotted as the number of seeds dispersed to a given distance.

for *Pgi-1* genotype and used to construct an experimental population. The experimental population comprised 91 plants spaced uniformly 0.6 m apart in a hexagonal array. Based on the behaviour of pollinators in natural populations, this array was chosen to be large enough for bees to forage without constraint. The centre of the population consisted of a core of seven plants homozygous for an electrophoretically rapidly migrating allele, *Pgi-1^r*. Along one margin of the population were plants homozygous for a slowly migrating allele, *Pgi-1^s*, while the body of the population consisted of plants homozygous for an electrophoretically intermediate allele, *Pgi-1^m*. Bees were allowed to forage freely on the plants in the experimental population for 3 days. During this time the distribution of pollinator flight distances between plants was recorded as a base for an inferred estimation of pollen gene flow. After the experimental period plants were returned to the greenhouse and the resulting fruits were marked. Of the F_1 progeny that resulted from pollinations during the experimental period 746 were assayed for *Pgi-1* genotype. Actual pollen gene flow was measured by detecting heterozygous F_1 progeny, *Pgi-1^{m/r}* if gene flow occurred from the centre of the population, or *Pgi-1^{m/s}* if gene flow occurred from the population margin.

Figure 1 presents the combined gene flow curves and the pollinator foraging flight distribution for the experimental population. Most of the pollinator moves and most gene flow are over very short distances. The actual movement of genes in the population is greater than would be inferred from the pollinator flight distribution alone. Mean gene movement, 1.82 ± 0.10 m, is almost twice as far as the mean pollinator flight distance, 0.97 ± 0.08 m. There were no significant differences between gene flow from the margin and from the centre of the population ($\chi^2 = 8.62$, $P > 0.05$). The shapes of both the pollinator flight distribution and the gene flow curve are leptokurtic, $g_2 = 11.7$ and 3.0 , respectively ($P < 0.05$). The curves differ significantly in relative proportions at each distance ($\chi^2 = 427.3$, $P < 0.05$). The disparity between the two curves is a consequence of pollen carryover from one plant to the next. Pollen carryover occurs when not all of the pollen a bee deposits on a stigma is from the flower just visited; some fraction comes from flowers visited before the most recent. Despite pollen carryover by pollinators, gene dispersal via pollen is still restricted, most gene movement being to neighbouring plants.

As gene movements by both pollen and seed are determinants of genetic neighbourhood size, the seed dispersal distribution was determined for *L. texensis*. Plants whose fruits were ripe and ready to dehisce were placed in an unobstructed area, and the actual distance of seed dispersal from parent plants was measured for 263 seeds (Fig. 2). The seed dispersal distribution is leptokurtic ($g_2 = 5.5$, $P < 0.05$), and the mean seed dispersal distance is 0.58 ± 0.04 m. Seed dispersal as well as pollen dispersal is also restricted; most seeds move but a slight distance from the parent.

Restricted gene flow within a population, such as that observed here, results in non-random mating within the population as a whole. Wright has defined the neighbourhood as that subunit of a population within which there is random mating

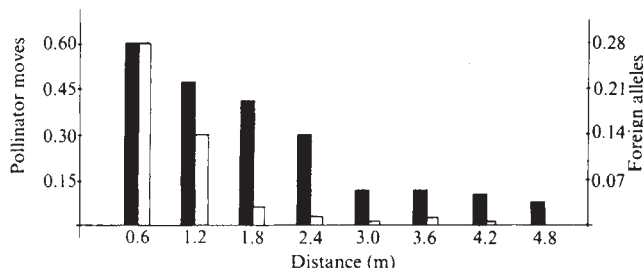


Fig. 1 Gene flow and pollinator foraging distances in *Lupinus texensis*. Gene flow (solid bars) is plotted as the frequency of foreign alleles (either *Pgi-1^r* or *Pgi-1^s*) detected in the F_1 progeny of plants located at a given distance. Pollinator moves (open bars) are plotted as the frequency of the total pollinator moves to a given distance.

(panmixia)³. As there is a significant difference between the estimated gene flow from pollinator foraging distances and actual gene flow, it is of interest to know how such differences influence neighbourhood size. Neighbourhood size, N_e , is defined as the number of individuals which compose a panmictic unit, and its value is estimated by:

$$N_e = 3.6D \left(\frac{\sum p^2}{2N_p} + \frac{\sum s^2}{N_s} \right)$$

where p is the dispersal distance of genes via pollen, s is the axial seed dispersal distance, N_p is the number of pollen dispersed genes, N_s the number of seeds and D is the flowering plant density⁴. Neighbourhood size estimates are generally derived most reliably from data of experimental populations. These estimates have meaning in natural populations as well, as the nature of gene flow is determined predominately by inherent characteristics of the species, such as its modes of pollination and of seed dispersal. Except at the extremes of density, gene flow is influenced only minimally by the distribution of individuals peculiar to a given site.

In the experimental population of *L. texensis*, neighbourhood size, based on gene flow estimated from pollinator movement, is 42.2 plants and neighbourhood area is 2.8 m². The neighbourhood size based on actual gene flow is more than twice as large, 95.4 plants, and the area is 6.3 m². Pollen carryover thus has a significant effect on actual neighbourhood size.

The distribution of pollinator flight distances provides only a minimal estimate of the pollen component of gene flow in *Lupinus texensis* and most likely, in other plant species as well. Actual gene flow distance somewhat exceeds that predicted from pollinator flight distribution alone. However, gene flow due to pollen movement is still very restricted spatially. In this study, no gene migration from one margin of the population to the other was detected. The restriction of gene flow is manifest in the small estimates of neighbourhood size and area. A large body of theoretical work predicts that such limited gene flow will greatly influence the genetic structure of populations^{5,6}. As natural populations of *L. texensis* are typically orders of magnitude greater in size than the estimated neighbourhood size—often larger than 10,000 individuals and covering several hectares—they are likely to be subdivided into many neighbourhoods. Such populations are not panmictic, and should exhibit such properties as significant genetic heterogeneity, isolation by distance, and possibly inbreeding.

I thank W. W. Anderson, W. J. Leverich and C. W. Birky Jr for their comments. This study was supported by NSF grants DEB 76-09709 and DEB 79-05198.

Received 21 September 1979; accepted 7 February 1980.

1. Bateman, A. *Heredity* **1**, 303–336 (1947).
2. Griffiths, D. J. *J. agric. Sci.* **40**, 19–38 (1950).
3. Wright, S. *Genetics* **31**, 39–50 (1946).
4. Levin, D. & Kerster, H. *Am. Nat.* **105**, 345–354 (1971).
5. Wright, S. *Proc. Am. phil. Soc.* **93**, 471–478 (1949).
6. Gregorius, H. *Theor. Population Biol.* **8**, 134–142 (1976).

Serotyping *Plasmodium falciparum* malaria with S-antigens

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The heat-stable proteins known as S-antigens which are associated with *Plasmodium falciparum* malarial infections in man show considerable serological diversity¹. Usually, different S-antigens appear in separate malarial episodes in individuals exposed to reinfection². The antigens have a worldwide distribution in endemic areas but the restricted number and characteristic specificity of S-antigens recovered from experimentally infected *Aotus* monkeys suggest that these antigens might provide suitable markers for serotyping isolates of *P. falciparum*³. Two kinds of evidence to support this idea are presented here. First, S-antigens which characterise an isolate of parasites are shown to retain their specificity over long periods of passage either *in vivo* or *in vitro*. Second, predictable mixtures of S-antigens can be recovered from cultures of *P. falciparum* after deliberately mixing isolates which each give rise to characteristic S-antigens.

The human isolates of *P. falciparum* described in this work were obtained by sampling at random acutely infected children attending the outpatients clinic at the MRC Laboratories in The Gambia, West Africa. Plasma from each blood sample was stored frozen until tested for S-antigen content by gel diffusion. S-antigens were identified by their stability to heating at 100 °C for 5 min⁴. Washed infected erythrocytes from each patient were cryopreserved in glycerol buffer at –196 °C and subsequently thawed and reconstituted by a simplified method⁵. The thawed infected erythrocytes were cultivated *in vitro* with human red cells of blood group A or O (ref. 6). The medium consisted of RPMI 1640 with 15% serum and was changed twice daily. The gas phase was 5% O₂, 7% CO₂, and 88% N₂. Pools of waste culture fluid were centrifuged at 950g to remove cellular debris and were concentrated 10-fold by negative pressure dialysis. Plasma samples were also collected from *Aotus*

monkeys infected with various named isolates of *P. falciparum*, through the courtesy of Dr W. Collins, C.D.C. Atlanta, Georgia. In other experiments, an isolate of *P. falciparum* was serially passaged through splenectomised squirrel monkeys (*Saimiri* sp.), in collaboration with Dr M. Hommel, Institut Pasteur, Paris.

The long-term stability of S-antigen expression was tested using the Ugandan Palo Alto line of *P. falciparum* which has two main S-antigens (Table 1). After more than 10 passages through *Aotus* monkeys, this line of parasites was cultivated *in vitro* in human erythrocytes for 1 month. The parasites were then passed serially through two previously infected (partially immune) *Aotus* monkeys which developed low-grade parasitaemias. The parasites which emerged were transferred by direct blood passage to splenectomised squirrel monkeys and underwent 29 serial transfers before propagation in cultures of human red cells for a further month. Finally the parasitised erythrocytes were inoculated into a squirrel monkey to produce an acute infection. The S-antigens in the plasma of this animal as well as from several of the previous squirrel and *Aotus* monkeys and also from concentrated culture fluids were compared serologically. No alteration was detected in the S-antigen specificities throughout these manipulations. Although selection of parasites

Table 1 Distribution of S-antigens in *Aotus*-adapted isolates of *P. falciparum*

Isolate	S-antigen specificities*						
	a	b	c	d	e	f	g
Malayan Camp	+	+	–	–	–	–	–
Ugandan Palo Alto	+	+	–	–	–	–	–
Salvador (St Lucia)	+	+	–	–	–	–	–
West African I	+	+	–	–	–	–	–
West African (Lagos)	–	–	+	+	(+)	†	–
Nigerian I	–	–	–	(+)	+	–	–
Haitian III	–	–	–	–	(+)	+	–
Haitian I	–	–	–	–	–	–	?‡
Cambodian I	–	–	–	–	–	–	?‡

* Specificities a and b correspond to S-antigens 7 and 8 in Fig. 3 in ref. 1.

† (), Trace only.

‡ Antiserum not available.

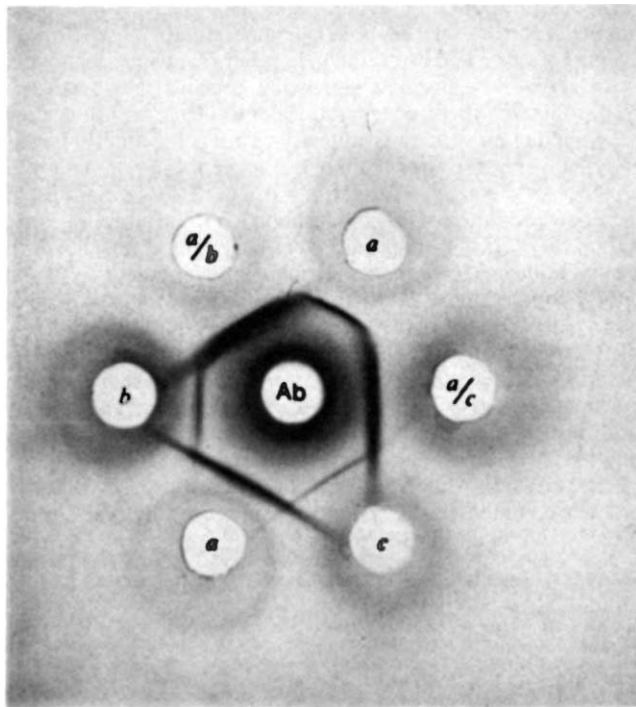


Fig. 1 Production of predicted S-antigens by three lines of *P. falciparum* cultivated *in vitro*. *a*, Plasma from *Aotus* monkey infected with Palo Alto line. *b*, *c*, Plasmas from two different human infections. *a/b* S-antigens from a mixed culture of *a* and *b* parasites. *a/c* S-antigens from a mixed culture of *a* and *c*, with *c* in a minority. *Ab*, Antiserum from an immune Gambian adult.

might have occurred at several points during the series of passages, it appeared that some of the original parasite type persisted. No new type was found despite screening with several heterospecific antisera known to react with a wide variety of S-antigens.

It is necessary to point out here that different S-antigens can be demonstrated in different lines of *P. falciparum* adapted to the *Aotus* monkey (Table 1). However, the frequency of antigen specificities 7 and 8, previously demonstrated in the Malayan Camp strain⁷, is unexpectedly high—four of nine *Aotus*-adapted isolates examined (44%). The same antigens occurred in only 16% of 50 malarious children tested at random. More surveys on these lines are desirable because recent immunological tests^{8,9} with *Aotus*-adapted lines of *P. falciparum* have been made on the assumption that lines with different names are serologically distinct.

It is not established clearly yet whether S-antigens are a direct product of the parasite genome or a host component modified in some unspecified way inside the parasitised erythrocyte. To demonstrate that different lines of *P. falciparum* express their characteristic S-antigens when grown in erythrocytes from a single source still would not indicate their parasite origin unambiguously. However, such an experiment would strongly support the hypothesis drawn from epidemiological and other studies that different S-antigens are characteristic of different isolates of parasites.

To carry out this experiment, plasma samples from 30 acutely infected Gambian children were screened to find appropriately reactive S-antigens and to select those with different specificities. Cryopreserved infected erythrocytes from the selected isolates were reconstituted and cultivated *in vitro*. Examination of concentrated culture fluids from the different lines confirmed that the expected S-antigens were present. Two of these recently derived human lines of *P. falciparum* were then mixed individually with the Palo Alto line to produce mixed cultures. Gel diffusion analysis of concentrated culture fluids

from each mixed culture showed that they now contained the predicted mixture of S-antigens (Fig. 1).

These studies suggest that S-antigens can be used as stable markers of isolates of *P. falciparum* grown either in experimental hosts or in cultures. The findings support our earlier hypothesis¹ that S-antigens reflect considerable diversity in natural populations of *P. falciparum* and that mixed infections with different types occur commonly. This last point has also been confirmed by the demonstration of mixed isozymes of parasite glycolytic enzymes in extracts of infected erythrocytes¹⁰. S-antigens, however, provide a much more extensive marker system than the isozyme and drug resistance differences described by others. Immunological experiments on selective destruction of parasites producing different S-antigens in mixed cultures might throw light on the question of strain specific immunity to *P. falciparum* for which there is evidence in experimental infections of man¹¹ and the *Aotus* monkey^{8,12}.

This work was supported by the Malaria Component of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

Received 26 November 1979; accepted 18 February 1980.

1. Wilson, R. J. M., McGregor, I. A., Hall, P., Williams, K. & Bartholomew, R. *Lancet* ii, 201–205 (1969).
2. Wilson, R. J. M., McGregor, I. A. & Hall, P. J. *Trans. R. Soc. trop. Med. Hyg.* 69, 460–467 (1975).
3. Wilson, R. J. M. & Voller, A. *Parasitology* 64, 191–195 (1972).
4. Wilson, R. J. M., McGregor, I. A. & Wilson, M. E. *Int. J. Parasit.* 3, 511–520 (1973).
5. Phillips, R. S. & Wilson, R. J. M. *Trans. R. Soc. trop. Med. Hyg.* 72, 643–644 (1978).
6. Trager, W. & Jensen, J. B. *Science* 193, 673–675 (1976).
7. Wilson, R. J. M. & Voller, A. *Parasitology* 61, 461–464 (1970).
8. Mitchell, G. H., Butcher, G. A., Richards, W. H. G. & Cohen, S. *Lancet* i, 1335–1338 (1977).
9. Reese, R. T. & Motyl, M. R. *J. Immun.* 123, 1894–1899 (1979).
10. Carter, R. & McGregor, I. A. *Trans. R. Soc. trop. Med. Hyg.* 67, 830–837 (1973).
11. Jeffery, G. M. *Bull. WHO* 35, 873–882 (1966).
12. Voller, A. & Richards, W. H. G. *Z. Parasitenk.* 21, 159–166 (1970).

Mouse C_μ heavy chain immunoglobulin gene segment contains three intervening sequences separating domains

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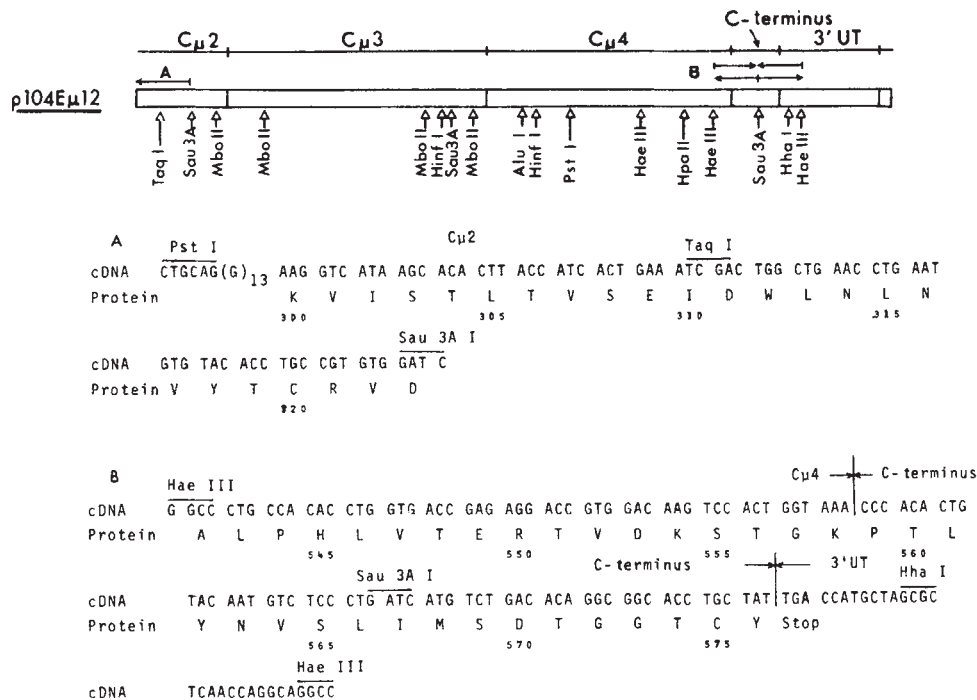
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The IgM molecule is composed of subunits made up of two light chain and two heavy chain (μ) polypeptides. The μ chain is encoded by several gene segments—variable (V), joining (J) and constant (C_μ)^{1,2}. The C_μ gene segment is of particular interest for several reasons. First, the μ chain must exist in two very different environments—as an integral membrane protein in receptor IgM molecules (μ_m) and as soluble serum protein in IgM molecules into the blood (μ_s). Second, the C_μ region in μ_s is composed of four homology units or domains ($C_{\mu 1}$, $C_{\mu 2}$, $C_{\mu 3}$ and $C_{\mu 4}$) of approximately 110 amino acid residues plus a C-terminal tail of 19 residues^{3,4}. We asked two questions concerning the organisation of the C_μ gene segment. (1) Are the homology units separated by intervening DNA sequences as has been reported for α (ref. 5), γ_1 (ref. 6) and γ_{2b} (ref. 7) heavy chain genes? (2) Is the C-terminal tail separated from the $C_{\mu 4}$ domain by an intervening DNA sequence? If so, DNA rearrangements or RNA splicing could generate hydrophilic and hydrophobic C-terminal tails for the μ_s and μ_m polypeptides, respectively. We demonstrate here that intervening DNA sequences separate each of the four coding regions for C_μ domains, and that the coding regions for the $C_{\mu 4}$ domain and the C-terminal tail are directly contiguous.

mapped are consistent with the coding sequence. There are no sites in the insert for *EcoRI*, *BamHI*, *KpnI* or *XbaI*. A and B show DNA sequences from p104E μ 12. Sequences were obtained by the method of Maxam and Gilbert¹⁷, with minor modifications using the 5' end-labelled restriction fragments indicated in the restriction map. Sequence A was determined in one direction only; nucleotides printed in lower case type were ambiguous and have been supplied from the coding requirements. Sequence B was determined in both directions and was unambiguous.

R-loop analyses using heavy chain mRNA from myeloma tumour M104E were performed and similar results were

The coding regions were located more precisely by restriction mapping (Fig. 2). We mapped *Hinf*I, and *Sau*3A sites in the region of the four C_μ domains in order to measure the sizes of the intervening sequences. A *Sau*3A fragment spanning the $C_{\mu 2}$ – $C_{\mu 3}$ junction is 331 base pairs long in the μ 12 cDNA clone and 620 base pairs long in the germ-line clone ChSp μ 7 (μ 7). Therefore, an intervening DNA sequence of 289 ± 15 base pairs exists between $C_{\mu 2}$ and $C_{\mu 3}$. A *Hinf*I fragment spanning the $C_{\mu 3}$ – $C_{\mu 4}$ junction is 122 base pairs long in the cDNA clone and 230 base pairs long in the germ-line clone, while a *Sau*3A–*Pst*I fragment is 147 and 254 base pairs long in the two clones, respectively. Accordingly, there are 108 ± 10 base pairs of intervening sequence between $C_{\mu 3}$ and $C_{\mu 4}$. The $C_{\mu 1}$ – $C_{\mu 2}$ junction was not available in a cDNA clone, but the entire



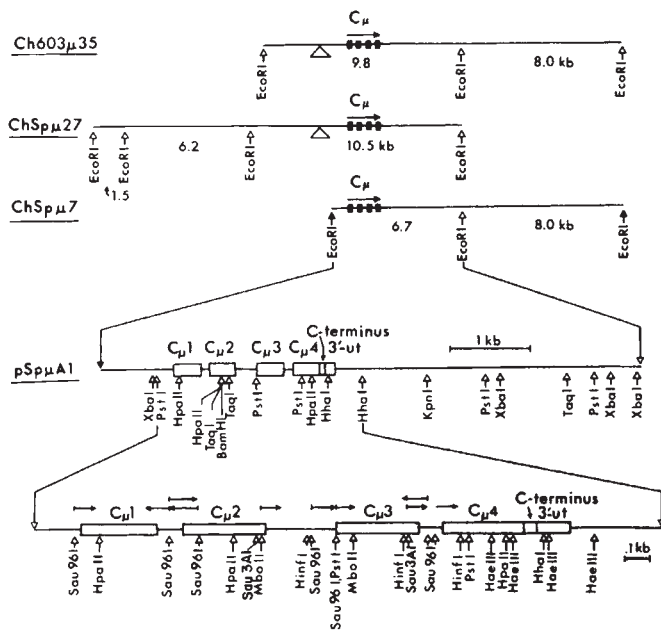


Fig. 2 Restriction maps of three C_μ genomic inserts in Charon 4A. Natural *EcoRI* sites are indicated by open arrow heads, synthetic ones by filled arrow heads. The 8-kilobase *EcoRI* fragment in $\mu 35$ may be a cloning artefact. The C_μ coding regions were located by R-loop and restriction enzyme mapping, as well as DNA sequencing. The direction of transcription, indicated by arrows above the coding regions, was determined by comparison of restriction enzyme sites in genomic clones to $p\mu 12$. Triangles indicate the approximate locations of deletions¹. The 6.7-kilobase *EcoRI* fragment from ChSp $\mu 7$ ($\mu 7$) was recloned by ligation into the *EcoRI* site of pBR322, generating plasmid pSp $\mu A1$. In the $\mu 7$ region shown by R-looping to contain the C_μ gene, digestion with restriction enzymes determined that $p\mu 12$ could be mapped onto pSp $\mu A1$ with intervening sequences between $C_\mu 2$, $C_\mu 3$ and $C_\mu 4$. The $C_\mu 1$ domain has been positioned by R-looping mapping and DNA sequencing. The lower portion of the figure depicts the sequencing strategy used to define precisely intervening sequence boundaries (Fig. 4). All *PstI*, *HhaI* and *HpaII* sites in the region of the C_μ gene are shown, together with those sites for other enzymes which were used for sequencing (arrows) and for identifying intervening sequences. These include the sites bounding those fragments described in the text which span the $C_\mu 2/C_\mu 3$, $C_\mu 3/C_\mu 4$, and $C_\mu 4/C$ -terminal-3' untranslated junctions.

intervening sequence between $C_\mu 1$ and $C_\mu 2$ from the genomic clone $\mu 7$ has been sequenced and is 107 base pairs long (J. R., unpublished results).

All of the boundaries of the coding and intervening sequences in the C_μ gene segment were sequenced using the strategy shown in Fig. 2. Figure 4 gives the sequences of the splice sites and shows that there is terminal redundancy about each of the intervening sequences. At the downstream splice sites between J_H and $C_\mu 1$, and between $C_\mu 1$ and $C_\mu 2$, the noncoding sequence is identical to the preceding coding sequence for 7 and 8 nucleotides, respectively, before the indicated splice points. The precise splice points can be designated according to the/GT...AG/rule⁹, and the junction sequences are then found to conform generally to the 'consensus' RNA splicing sites (Fig 2)⁹⁻¹². The intervening sequences occur in codons 127, 230, 340 and 446. These are identical to the C_μ domain boundaries as far as they could be determined from protein sequence homologies⁴.

Restriction mapping also indicates that the $C_\mu 4$ coding region and the C-terminal tail are not separated by an intervening sequence but are continuously encoded in the germ-line DNA. This junction is spanned in $p\mu 12$ by a *PstI*-*HhaI* fragment (Fig. 1), which is 297 base pairs long according to the coding requirements. The corresponding fragment from a subclone of $\mu 7$ (Fig. 2) co-migrates with the $\mu 12$ fragment to an accuracy of ± 15 base pairs. This fragment was isolated from the $\mu 7$ sub-

clone and digested separately with *HaeIII*, *Sau3AI*, and *HpaII*. The fragments observed were identical to those predicted from the map of the $\mu 12$ cDNA clone (Fig. 1) to an accuracy of ± 6 base pairs. In additions, the $C_\mu 4$ -C-terminal junction is spanned in $\mu 12$ by a completely sequenced 132-base pair *HaeIII* fragment. The *HaeIII* fragment from the corresponding region of $\mu 7$ co-migrates with this to within ± 2 base pairs. Thus detailed restriction analyses demonstrate that within the limits of these analyses (± 2 base pairs) there is not an intervening DNA sequence between the $C_\mu 4$ domain and the C-terminal tail.

Our current observations on the C_μ genomic clones, in conjunction with previous studies on $C_\alpha 5$ and $C_\gamma 1$ and $C_\gamma 2b$ genomic clones, suggest that all immunoglobulin C_H genes will contain intervening sequences separating the regions coding for structural domains. Although the function of intervening sequences in eukaryotic genes remains unclear, their positioning precisely at the interdomain boundaries of immunoglobulin C_H genes suggests that the positions of the intervening DNA sequences may have some role in the evolution of immunoglobulin genes. As individual immunoglobulin domains probably encode dis-

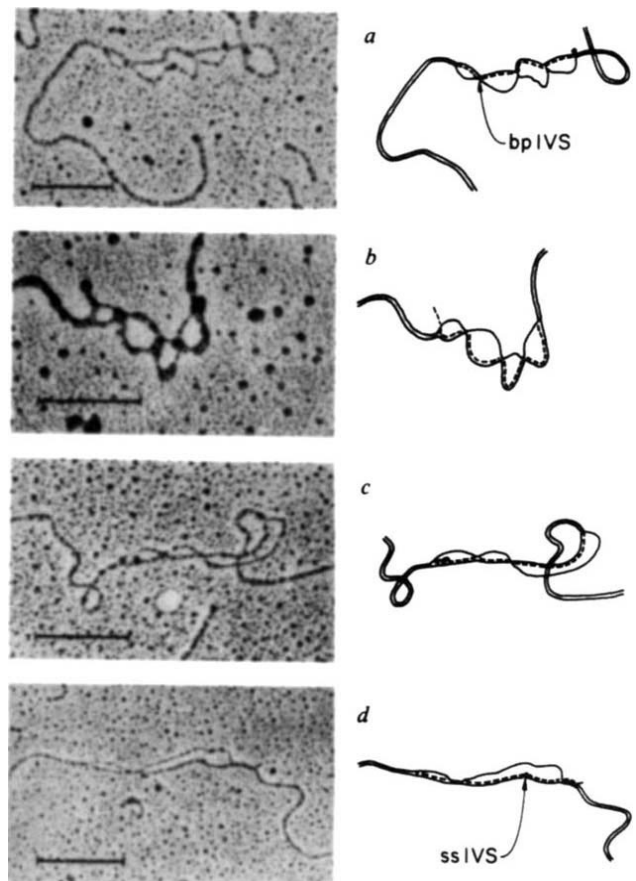


Fig. 3 Electron micrographs of R-loops formed from genomic C_μ clones and purified M104E mRNA. Cloned DNA was photochemically cross-linked with 4,5',8-trimethylpsoralen to produce an average of one cross-link every 4 kilobases¹⁸ before incubation with M104E mRNA in R-loop conditions¹⁹. R-loops were either spread immediately from a hyperphase of 70% (v/v) three times recrystallised formamide or were fixed with 1 M glyoxal at 12 °C for 2 h before spreading. Both procedures gave similar results. *a*, *b*, R-loops on Ch603 $\mu 35$ DNA. The arrow indicates the location of a typical base-paired intervening sequence (bpIVS). Both molecules show four C_μ coding regions interrupted by three base-paired intervening sequences. *c*, *d*, R-loops on ChSp $\mu 27$ DNA. Molecule in *c* shows two base-paired intervening sequences, that in *d* shows one base-paired intervening sequence and one single-stranded intervening sequence (ssIVS), indicated by arrow. In both molecules *c* and *d*, a third intervening sequence was not clearly observed. mRNA coding for the V region remains unhybridised and is visible in extended form in *b* and *c*. Scale bars, 0.5 μm .

	Junctions	Intervening sequences	
Fig. 4 Junctional sequences in the germ-line C_μ gene. *The location and sequence of J_{H107} in ChSp μ 27 is from ref. 2.	$J_{H107}^*/C_{\mu 1}$	ValSerSer GTCTCTCAGCTAAGCTGGCTT---	7.5 $\pm$ 0.8 kb -----GTCTCTCAGCTAAGCTGCTG
	$C_{\mu 1}/C_{\mu 2}$	ProIlePro CCCATTCCAGCTAAGAACCAAA---	107 bp ---ACCTTGACCTTTCATTCCAGCTGTCGCA
	$C_{\mu 2}/C_{\mu 3}$	CysAlaAla TGTGCTGCCAGTGTAGTGGCTGG---	289 $\pm$ 15 bp ---CAGTGTCTCTTGACTGCAGGTCCTCC
	$C_{\mu 3}/C_{\mu 4}$	LysProAsn AAACCAATGTAGGTATCCCCC---	108 $\pm$ 10 bp ---ACTACTGTCTTCATTACAGAGGTGCAC
	Consensus sites	5' AGGTAAGTA-----TTTTTTTTTCTTNCAGG	3'

tinct functions, the presence of intervening DNA sequences at the domain boundaries may facilitate the rearrangement of domain coding regions and thereby generate new combinations of heavy chain domains for selection, thereby speeding up the evolution of immunoglobulin genes^{8,13}. Alternatively, it also has been proposed that intervening DNA sequences inhibit recombination and, accordingly slow the evolution of eukaryotic genes^{14,15}.

Our studies on the organisation and structure of the C_μ gene segment place several constraints on models for the difference between μ_m and μ_s chains. First, Southern blot analyses of embryo or germ-line DNA with the μ 12 probe show only strongly hybridising bands corresponding to a single C_μ gene segment which is present in the μ 35, μ 27 and μ 7 clones. This is true for digests with *Eco*RI or *Hinc*II (ref. 1), as well as for *Bam*HI or *Hha*I (M. D., unpublished results). We have isolated 10 independent genomic clones which hybridise to μ 12, and all of these seem to contain the same C_μ gene segment (M. D., K. C. and P. E., unpublished results). Thus, these restriction mapping and gene cloning results strongly suggest that there is only one C_μ gene segment in the BALB/c genome. If so, the μ_s and μ_m chains must be encoded by the same C_μ gene segment. Second, a DNA rearrangement during B-cell development to generate alternative $C_{\mu m}$ and $C_{\mu s}$ gene segments is unlikely. In B-cell development, the μ_m chain is expressed before the μ_s chain. Thus, a putative DNA rearrangement should alter the 3' structure of the expressed $C_{\mu s}$ gene segment. However, our results show that the 3' end of the C_μ gene in the μ 12 cDNA clone is identical to the 3' end of the C_μ gene in the μ 7 germ-line clone, thus ruling out the possibility of a DNA rearrangement at the 3' coding region of the C_μ gene segment. Finally, we can rule out a simple post-translation cleavage of a larger μ_m chain to create the μ_s chain because the μ_s coding sequence is followed immediately by a stop codon (Fig. 1).

Two models to explain the origins of μ_m and μ_s still appear plausible: (1) the μ_m chain is generated from the μ_s chain by a novel type of post-translational modification; and (2) a different COOH-terminal coding region for the μ_m chain does exist. A large nuclear RNA transcript could give rise either to μ_s mRNA or, alternatively, to μ_m mRNA by RNA termination, cleavage and/or splicing. We are currently studying the μ RNAs from a B-cell lymphoma which produces only membrane IgM to test these models.

This work was supported by NSF grant PCM 76-81546 and NIH grants CA 12800 and AI 13410. M.D., P.E. and D.L. are supported by NIH Training Grant GM 07616. K.C. is supported by NIH Fellowship GM 05442.

- Sakano, H. *et al.* *Nature* **277**, 627 (1979).
- Kataoka, T., Yamawaki-Kataoka, Y., Yamagishi, H. & Honjo, T. *Proc. natn. Acad. Sci. U.S.A.* **76**, 4240 (1979).
- Davis, M., Early, P., Calame, K., Livant, D. & Hood, L. in *Eukaryotic Gene Regulation, ICN-UCLA Symposium* (eds Axel, R., Maniatis, T. & Fox, C. F.) 393 (Academic, New York, 1979).
- Breathnach, R., Benoist, C., O'Hare, K., Gannon, F. & Chambon, P. *Proc. natn. Acad. Sci. U.S.A.* **75**, 4853 (1978).
- Seif, I., Khoury, G. & Dhar, R. *Nucleic Acids Res.* **6**, 3387 (1979).
- Rogers, J. & Wall, R. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
- Lerner, M. R., Boyle, J. A., Mount, S. M., Wolin, S. L. & Steitz, J. A. *Nature* **283**, 220-224 (1980).
- Gilbert, W. *Nature* **271**, 501 (1978).
- Tiemeier, D. C. *et al.* *Cell* **14**, 237 (1978).
- Nishioka, Y. & Leder, P. *Cell* **18**, 875 (1979).
- Roychoudhury, R., Jay, E. & Wu, R. *Nucleic Acids Res.* **3**, 101 (1976).
- Maxam, A. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **74**, 560 (1977).
- Kaback, D., Angerer, L. & Davidson, N. *Nucleic Acids Res.* **6**, 2499 (1979).
- Thomas, M., White, R. L. & Davis, R. W. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2294 (1976).

Anomalous phenotype in thymic acute lymphoblastic leukaemia

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Malignant blast cells in the various forms of acute leukaemia seem to be 'frozen' at early stages of haematopoietic and lymphoid cell maturation. These blasts express relatively stable morphological, enzymatic and antigenic phenotypes which may largely represent the normal gene products of the corresponding precursor cells¹⁻⁴. A number of findings support these suggestions. The phenotype of the common form of acute lymphoblastic leukaemia (cALL) corresponds to the phenotype of normal small non-T, non-B cells of lymphoid morphology detected in low numbers in the normal and regenerating infant bone marrow^{4,5}. Also, the phenotype of blast cells in Thy-ALL corresponds approximately to that of cortical thymocytes or their immediate precursors⁶⁻⁹. Leukaemia-specific (virus-induced or mutant) changes or aberrant expression of normal gene products may also occur in leukaemia. To describe such phenomena, detailed single cell studies comparing leukaemic cells with their appropriate normal (frequently very rare) counterparts are needed. In this study we have performed such a comparative study and find that the human Thy-ALL blasts express unexpectedly high amounts of HLA-A, -B and -C antigens: the sensitive single cell assays used failed to find normal cells (in infant and fetal thymus, and in bone marrow) which express the exact phenotype of Thy-ALL blasts.

Both cALL and Thy-ALL blast cells express large amounts of terminal deoxynucleotidyl transferase (TdT). This nuclear enzyme can be labelled by purified rabbit antibodies to TdT in

Received 2 November 1979; accepted 13 February 1980.

- Davis, M. M. *et al.* *Nature* **283**, 733 (1980).
- Early, P. W., Huang, H. V., Davis, M. D., Calame, K. & Hood, L. *Cell* (in the press).
- Putnam, F. W., Florent, G., Paul, C., Shinoda, T. & Shimizu, A. *Science* **182**, 287 (1973).
- Kehry, M., Sibley, C., Furnham, J., Schilling, J. & Hood, L. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2932 (1979).
- Early, P. W., Davis, M. D., Kaback, D., Davidson, N. & Hood, L. *Proc. natn. Acad. Sci. U.S.A.* **76**, 857 (1979).

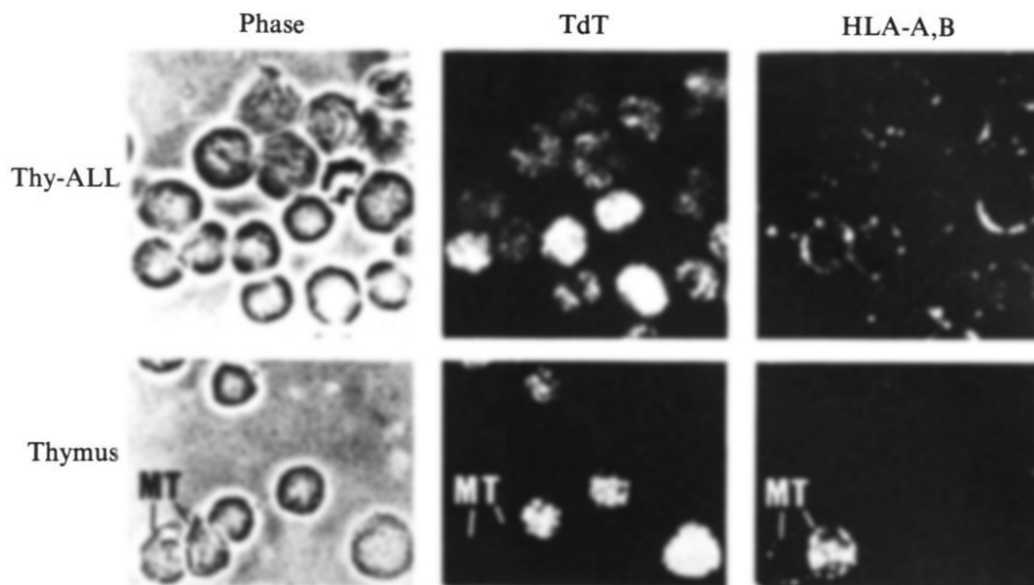


Fig. 1 Expression of HLA-A, -B and -C antigen and terminal transferase (TdT) on thymic ALL (leukaemic) blast cells and on normal thymocytes. Cells were incubated with W6/32 antibody (detecting core determinants of HLA-A, -B antigens)¹¹, washed and labelled with goat anti-mouse Ig-TRITC (red). The cells were smeared in a cytocentrifuge and restained for nuclear TdT by purified rabbit anti-TdT antibody¹⁰ followed by washing and a goat-anti-rabbit-Ig-FITC second layer (green). The preparations were photographed by filters selective for TRITC (HLA-A, -B and -C) and FITC (TdT). MT: putative medullary thymocytes. Additional studies have shown that in both Thy-ALL and thymocyte suspensions >95% of cells were strongly positive with anti-T cell serum (HuTLA⁺) and negative with anti-Ia antisera (Ia⁻)⁵.

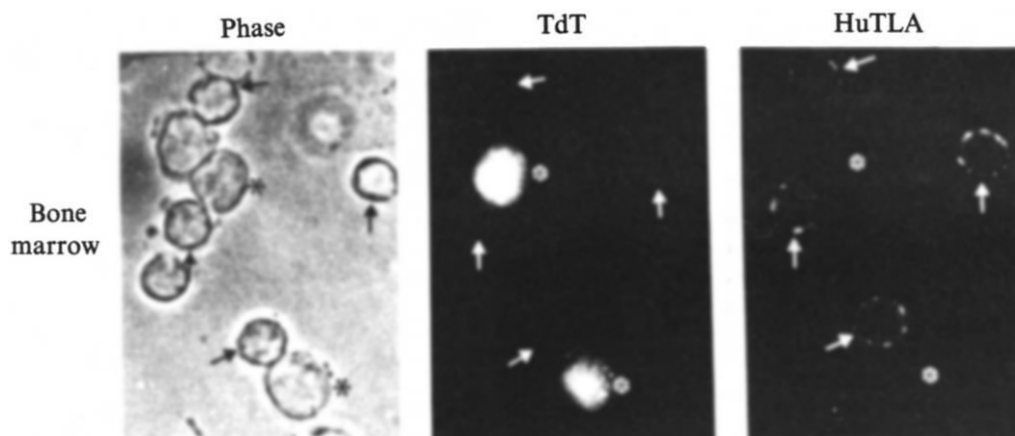


Fig. 2 In the normal human bone marrow T lymphocytes (HuTLA⁺, TdT⁻; arrows) and TdT⁺ precursor cells (HuTLA⁻, TdT⁺; asterisks) represent different populations. Cells were incubated with rabbit anti-HuTLA, labelled with goat-anti-rabbit-TRITC (red) and smeared. This was followed by restaining with rabbit anti-TdT and goat-anti-rabbit-FITC (green). Additional studies have shown that TdT⁺ bone marrow cells are HLA-A, -B and -C positive (<99%) and mostly Ia⁺ (>85%). None of these cells express HTA-1 (thymocyte-specific) antigen (see ref. 5).

indirect immunofluorescence (IF) test using fluorescein (FITC)-conjugated goat anti-rabbit immunoglobulin as second layer¹⁰. The test can conveniently be combined with other IF tests for various membrane or cytoplasmic antigens using tetra-rhodamine (TRITC)-labelled reagents (Table 1) and has been used both for the diagnosis of leukaemia and for the analysis of early lymphocyte maturation in man^{5,8}. Here, the comparative single cell assays on leukaemic and normal TdT-positive populations indicate that a large proportion of Thy-ALL blasts show anomalous phenotypic characteristics which could not be observed on normal TdT positive infant cells and fetal thymocytes.

The first relevant point was shown in suspensions of normal infant thymus labelled for nuclear TdT and core determinants of HLA-A, -B and -C antigens (using W6/32 monoclonal antibody; Fig. 1). No TdT⁺ cells had detectable HLA-A, -B and -C antigen in over 2,000 thymocytes analysed. These TdT⁺ cells

were cortical thymocytes expressing HTA-1, which is a cortical thymocyte-specific antigen (a possible homologue of mouse TL antigen¹²). Then the study was extended to the relatively rare (1–3%) TdT⁺ lymphoblasts in the infant thymus. These cells express only moderate amounts or no HTA-1 (ref. 8). When stained with Giemsa, these large blasts show a dark blue cytoplasm (putative infant pro-thymocytes). More than 400 of these TdT⁺ blasts were analysed but no HLA-A, -B, or -C positive blast cells were seen. On the other hand TdT⁻ (medullary; HTA-1⁻) thymocytes were brightly HLA-A, -B and -C positive. Thus the expression of TdT and HLA-A, -B and -C in normal thymocytes seems to be mutually exclusive for all the cells of the human thymus.

In marked contrast, in all six cases of Thy-ALL studied as well as in the Thy-ALL cell line studied (HPB-ALL; see phenotypic characteristics in Table 1) TdT⁺ blast cells expressed moderate to large amounts of HLA-A, -B and -C (Fig. 1). This was in spite

Table 1 Analysis of Thy-ALL blasts in comparison with normal infant and fetal thymocytes and TdT⁺ bone marrow cells

Markers	Normal child thymus		Fetal thymus	Normal child bone marrow	Thy-ALL patients						Thy-ALL line HPB-ALL	
	All cells	Large cells			1	2	3	4	5	6		
% Of cells in the total population												
TdT ⁺	65	3	6‡	4	82	92	84	98	95	35	54	
E-rosette	92		85	5	55§	50	<5	NT	<1	52	37	
% Of cells within the TdT ⁺ population*												
HLA-A,B,C†	<0.1	<0.1	<1	>99	>95	88	90	90	90	80	98	
HuTLA	97	88	+	<0.1	>95	>95	>95	>95	>95	>95	98	
Ia-like	0	0	—	93	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	
HTA-1	88	62	+		16	32	5	0.5	0.2	<0.1	47	
HLe-I	94	50	NT	34	35	4	68	2	27	<1	30	

Viable cell suspensions were labelled with various antisera (see below) in indirect immunofluorescence test using tetra-rhodamine (TRITC)-labelled second layers. Of the membrane-labelled cells smears were made in a cyto-centrifuge and fixed for 30 min in cold methanol. These fixed smears were incubated with rabbit anti-TdT antibodies (eluted from TdT antigen column; ref. 10), washed and stained with goat anti-rabbit immunoglobulin labelled with fluorescein (FITC). The nuclear TdT staining (FITC) is easily distinguishable from the membrane staining (TRITC). The study was focused on the TdT⁺ cells in the different samples and the TdT[−] cells were ignored. The samples studied contained different proportions of TdT⁺ cells (as shown). NT, not tested.

*Results are expressed as cells stained with membrane markers within the TdT⁺ population studied.

†The antibodies used (as first layers) to detect membrane markers were as follows: anti-HLA-A, -B and -C antibody was a mouse monoclonal reagent, W6/32, detecting a core HLA-A, -B and -C antigenic determinant, and was used as culture supernatant at 1:10 final dilution; anti-HuTLA serum was a rabbit anti-monkey thymocyte serum absorbed with human red cells, B lymphoid and myeloid leukaemic cells⁵ and used at 1:25 final dilution; anti-Ia-like serum was made in chickens against purified human Ia-like (p28,33) antigens⁵ and was used at 1:25 dilution; anti-HTA-1 antibody was a mouse monoclonal antibody NA1/34, specifically reactive with human cortical thymocytes¹², and used as peritoneal exudate at 1:400 final dilution⁸; anti-HLe-I reagent was also a monoclonal antibody (2D1), strongly reactive with thymocytes and T and B lymphocytes, while only weakly reactive with myeloid cells. This latter antibody was used in the form of culture supernatants at 1:5 final dilution⁸. The second layers (goat-anti-mouse-Ig-TRITC, goat-anti-rabbit-Ig-TRITC and sheep-anti-chicken-TRITC) were from Nordic Labs. Both first and second layers were titrated and used at saturating condition with no detectable nonspecific staining (see details in ref. 8).

‡In the fetal thymus the few TdT⁺ cells stain extremely weakly. These cells are small HLA-A,B[−], HTA-1⁺ thymocytes¹⁷.

§Some of these samples were tested 2 d after being obtained; the E⁺ values could therefore be artificially low.

||Weak staining.

of the fact that a variable proportion of the blast cells also expressed HTA-1.

As there was a marked discrepancy between the staining pattern of infant thymocytes and Thy-ALL blasts, we have investigated normal fetal thymocytes. The possibility that Thy-ALL might derive from fetal thymocytes has been raised by Gatien *et al.*¹³ and Stein *et al.*⁷ who showed that these cell types, unlike mature cortical thymocytes, carry receptors for activated C3 and form EAC rosettes. Nevertheless, in the three fetal thymus samples (at 14, 17 and 19 gestational weeks) simultaneously analysed for nuclear TdT and membrane HLA-A, -B and -C expression no cells expressed both markers. The vast majority of cells (90–95%, including most HTA-1⁺ cells) were TdT[−]. These results were in accord with the low or undetectable TdT values observed in the same fetal human thymus samples by the biochemical assay (G. J., K. Ganeshaguru and A. V. Hoffbrand, unpublished observation) and with observations in chicken¹⁴, mice and rats¹⁵, which also showed that fetal thymocytes are TdT[−].

In the final part of the study we postulated that perhaps Thy-ALL blast cells express the phenotypic characteristics of rare bone marrow (BM) cells. BM samples from 5–12-year-old children with no haematological malignancy were analysed with various double markers. The phenotypes of these TdT⁺ BM cells were, again, different from that of both Thy-ALL blasts and thymocytes. Unlike thymocytes and Thy-ALL blasts, BM TdT⁺ cells failed to express HuTLA and HTA-1 (Fig. 2; see also ref. 5). All TdT⁺ BM cells (>99%) were brightly stained for HLA-A, -B, and -C; and the vast majority (>85%) were also brightly stained for Ia-like antigens (Ia⁺). This agrees with previous results⁵, suggesting that the phenotypic characteristics of TdT⁺ BM cells were similar to the phenotype of cALL blasts and different from those of Thy-ALL blasts.

In conclusion, our results indicate that human leukaemic blasts and cell lines of Thy-ALL phenotype (HuTLA⁺, E⁺, Ia[−], HTA-1⁺) have an unexpected expression of HLA-A, -B, and -C antigens when compared to small cortical thymocytes or putative large TdT⁺ thymic prothymocytes. There are at least three possible interpretations of our findings. It is possible that even the sensitive single cell assays used are unable to detect TdT⁺,

brightly HuTLA⁺, HLA-A, -B and -C⁺, Ia[−] bone marrow precursor cells (possibly bone marrow prothymocyte) from which Thy-ALL might develop because these cells are exceedingly rare (<10^{−4}). It is also conceivable that the generation of HLA-A, -B and -C antigens on cortical thymocytes is associated with the malignant change (for example the rare HLA-A, -B, and -C⁺ thymic cells might be predisposed to undergo a malignant transformation) and therefore most Thy-ALL show this peculiar phenotype. It seems more likely, however, that within the intact thymic cortex microenvironmental effects modulate (that is, they actively suppress) the expression of HLA-A, -B and -C antigens on normal cortical thymocytes as part of the physiological interplay between epithelial and lymphoid elements. This possibility is in line with the experimental observations that only during this special cortical development stage do T-lineage cells lack the easily detectable HLA, -B and -C antigens^{16,17}. Thus, although Thy-ALL blasts may have originated from a thymic precursor cell (HuTLA⁺, TdT⁺, Ia[−]), they exhibit HLA-A, -B and -C antigens perhaps due to the loss of thymic microenvironmental control during the disseminated phase of the disease.

We thank Dr P. Beverley (ICRF Human Tumour Immunology Unit, University College Hospital, London) for 2D1 antibody.

Received 27 November 1979; accepted 7 February 1980.

1. Sachs, L. *Nature* **274**, 535–539 (1978).
2. Metcalf, P. & Moore, M. A. S. in *Haemopoietic Cells* (North Holland, Amsterdam, 1971).
3. Salmon, S. E. & Seligmann, M. *Lancet* **ii**, 1230–1233 (1974).
4. Greaves, M. F. & Janossy, G. *Biochim. biophys. Acta* **516**, 193–230 (1978).
5. Janossy, G. *et al. J. Immun.* **123**, 1525–1529 (1979).
6. Mills, B., Sen, L. & Borella, L. *J. Immun.* **115**, 1038–1044 (1975).
7. Stein, H. *et al. Int. J. Cancer* **17**, 292–295 (1976).
8. Bradstock, K. F. *et al. J. natn. Cancer Inst.* (in the press).
9. Reinherz, E. L., Kung, P. C., Goldstein, G., & Schlossman, S. F. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
10. Bollum, F. J. *Proc. natn. Acad. Sci. U.S.A.* **72**, 4119–4122 (1975).
11. Barnstable, C. J. *et al. Cell* **14**, 9–21 (1978).
12. McMichael, A. J. *et al. Eur. J. Immun.* **9**, 205–210 (1979).
13. Gatien, J. G., Schneeburger, E. E. & Merlar, E. *Eur. J. Immun.* **5**, 312–317 (1975).
14. Sugimoto, M. & Bollum, F. J. *J. Immun.* **122**, 392–397 (1979).
15. Gregoire, K. E., Goldschneider, I., Barton, R. W. & Bollum, F. J. *J. Immun.* **123**, 1347–1357 (1979).
16. Brown, G., Biberfeld, P., Christensen, B. & Mason, D. Y. *Eur. J. Immun.* **9**, 272–275 (1979).
17. Janossy, G. *et al. J. Immunol.* (in the press).

Nonsteroidal compounds which bind epididymal androgen-binding protein but not the androgen receptor

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In the testis of most mammals the Sertoli cells of the seminiferous tubules secrete into the lumen an androgen-binding protein (ABP). This glycoprotein is of interest because it reflects Sertoli cell function and responsiveness to follicle-stimulating hormone¹. In addition, ABP binds testosterone produced by the Leydig cells and the hormone-protein complex reaches the epididymis² where it could play a role in sperm maturation³⁻⁶. ABP is distinct from the androgen receptor, an intracellular protein which binds testosterone and its hormonally active metabolite 5 α -dihydrotestosterone (DHT) in target tissues⁷. In some species, but not the rat, a testosterone-oestradiol binding globulin (TeBG) circulates in blood⁸. Although radioimmunoassays for TeBG⁹ and ABP¹⁰ have been developed, most methods for assaying these proteins and the androgen receptor rely on their ability to bind ³H-labelled androgens. As all three proteins bind testosterone and DHT, accurate assay of any one protein awaits development of a specific label. A specific ligand for ABP would also be very valuable for further study of the physiologic role of ABP. We¹¹ and others¹² have investigated earlier suggestions² that the binding sites on ABP and the androgen receptor have different steroid specificities. Steroids were found that have a higher affinity for ABP than for the receptor. We now report on dicyclohexane derivatives which bind with high affinity to the testosterone-binding site on ABP but do not interact with the androgen receptor.

The dicyclohexane derivatives shown in Table 1 were synthesised by catalytic perhydrogenation of diethylstilboestrol (III, V) or of mesohexestrol (IV). Chromic oxidation of these diols yielded the corresponding diketones (I, II). All compounds were purified and characterised as part of a project on the androgenic activity of nonsteroidal molecules¹³. We first studied their effect on the interaction of androgens with ABP from rat epididymis. Specific binding was determined by a charcoal adsorption assay which we have shown to give results identical to those obtained by the classical procedure of steady-state polyacrylamide gel electrophoresis¹¹. As illustrated in Fig. 1a, compounds I, II and III could inhibit completely the binding of DHT to ABP. This effect appeared to be specific since compounds IV and V at a 2,000-fold excess produced less than 20% inhibition. The question of whether the inhibition was competitive or not was then investigated. ABP binding of DHT was studied at increasing concentrations of the latter in the presence of fixed concentrations of the analogues. Lineweaver-Burk plots of data obtained at equilibrium confirmed that DHT interacts with a single class of sites as demonstrated earlier by Scatchard analysis¹¹ and suggested that the nonsteroidal compounds interfere with DHT binding by competitive inhibition (Fig. 1b). The equilibrium affinity constants calculated from these plots are listed in Table 1. In other experiments, 1 μ M nonradioactive DHT or compound I was added to preformed

³H-labelled DHT-ABP complex. In both cases, binding was completely reversible; the time-dependent decrease in specifically-bound radioactivity followed the same first-order kinetics (half life 5.8 min) with compound I as with DHT. Thus, the properties of the binding site for the natural androgen were not modified by the presence of compound I.

The effect of the dicyclohexane derivatives on the binding of androgens to their intracellular receptor protein was quite different. Cytosol from rat ventral prostate was incubated with ³H-labelled methyltrienolone (RU 1881), a specific ligand for the androgen receptor¹⁴. In conditions where testosterone and

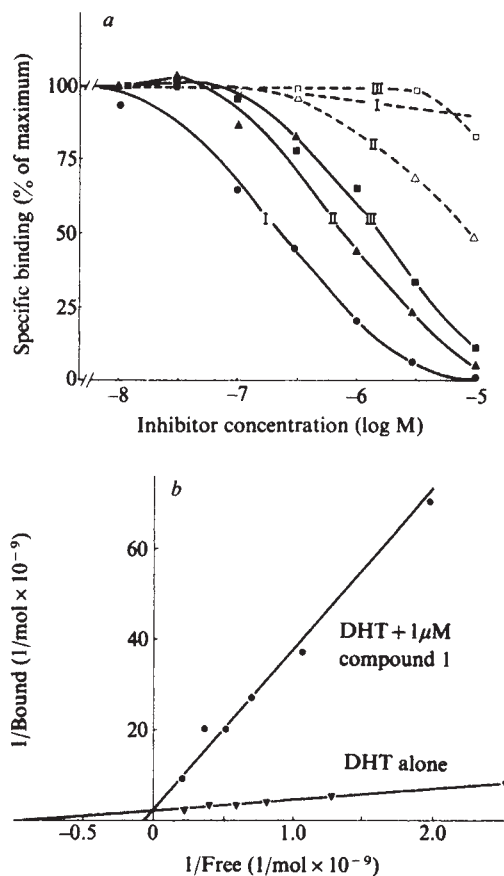


Fig. 1 Binding of dicyclohexane derivatives to ABP (closed symbols) and to the androgen receptor (open symbols). Epididymis and ventral prostates obtained from Wistar rats 24 h after castration were homogenised at 0 °C in 20 mM Tris-HCl buffer (pH 7.4) containing 1.5 mM EDTA and 10% (v/v) glycerol without (epididymis) or with (prostate) 0.25 mM dithiothreitol. Homogenates were centrifuged at 200,000g for 60 min at 0 °C to yield the cytosol. Androgen receptor in epididymis cytosol was inactivated by freezing (-20 °C) and thawing (30 °C, 30 min) in the absence of dithiothreitol, conditions to which ABP is insensitive¹¹. **a**, Epididymis cytosol was incubated for 3 h with 1.5 nM ³H-DHT in the presence of the indicated concentrations of inhibitors (see Table 1 for nomenclature) and specific binding to ABP was determined as described¹¹. Briefly, unbound hormone was removed from the 0.4 ml incubation mixtures by adding 0.2 ml of activated charcoal suspension (Norit A 50 mg ml⁻¹, dextran 5 mg ml⁻¹) and sedimenting the charcoal after 2 s of agitation. Nonspecific binding was calculated from parallel incubations containing a 500-fold excess of nonradioactive DHT. Prostate cytosol was incubated with 2.5 nM ³H-methyltrienolone for 16 h for determination of binding to the androgen receptor¹¹, in absence or presence of the inhibitors. Specific binding is expressed as a percentage of maximum binding seen in the absence of inhibitors (7,100 d.p.m. per ABP assay and 7,340 d.p.m. per receptor assay). Nonspecific binding was 10 and 27% of total binding for ABP and the receptor, respectively. **b**, Double-reciprocal plot of ABP binding at increasing ³H-DHT concentrations in the absence or presence (1 μ M) of inhibitor.

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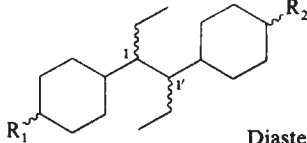
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DHT completely inhibited methyltrienolone binding, none of the compounds except II had any effect. Compound II displayed some inhibition at high concentrations (Fig. 1a). From double-reciprocal plots, this inhibition was found to be competitive.

Thus, compounds I and III specifically bind to ABP but not to the androgen receptor. Androgens are known to bind to the glucocorticoid receptor, in which system they behave as antagonists¹⁵. However, none of the nonsteroidal compounds bound to the glucocorticoid receptor from rat hepatoma cells. Unlike diethylstilboestrol from which it is derived compound I, which has the highest affinity for ABP, did not bind to the oestrogen receptor from rat uterus.

Since ABP has many characteristics in common with TeBG¹⁶, we wondered whether the nonsteroidal compounds also bound to TeBG. As this protein is not found in the rat, the test was performed on human TeBG. Human male plasma was diluted 10-fold with 20 mM Tris-HCl, 1.5 mM EDTA, 10% (v/v) glycerol, pH 7.4 and agitated for 45 min at room temperature with 1% (w/v) activated charcoal to remove endogenous steroids. After centrifugation the supernatant was incubated with 5 nM ³H-labelled DHT for 3 h without or with 10 μ M nonradioactive DHT and specific binding was determined as described for ABP. A 2,000-fold excess of compound III was required for inhibition of the binding of DHT to TeBG by 30%.

Table 1 Dicyclohexane derivatives studied

Compound			Diastereo-isomerism at C-1,1'	K_a (M ⁻¹)
	R ₁	R ₂		
I	= 0	= 0	Racemic	12.0×10^6
II	= 0	= 0	Meso	4.8×10^6
III	e OH, a H	a OH, e H	Racemic	3.5×10^6
IV	e OH, a H	e OH, a H	Meso	0.8×10^6
V	e OH, a H	e OH, a H	Racemic	0.6×10^6

e, equatorial; a, axial (see ref. 13). K_a is the equilibrium association constant (0 °C) for rat ABP calculated for double-reciprocal plots as in Fig. 1.

In these conditions, the other compounds had no effect while, as expected, oestradiol completely inhibited DHT binding to TeBG. Thus, in our assay systems, compound I bound strongly to ABP but not to TeBG nor to the androgen receptor. These results, however, do not allow immediate predictions concerning binding of the nonsteroidal analogues to the controversial¹⁷⁻²⁰ human ABP as it is known that ABPs from different species differ in steroid specificity²¹.

The data presented here confirm that the androgen-binding sites on ABP and the androgen receptor have different specificities. We have found dicyclohexane derivatives which display an absolute specificity for ABP when tested in parallel for androgen receptor binding. Steroidal and nonsteroidal ligands which specifically inhibit binding of androgens to ABP may be of interest for studying regulation of male fertility.

G.G.R. is Maître de Recherches of the FNRS (Belgium). We thank M. A. Gueuning and Th. Lambert for technical assistance. This research was supported in part by the Roussel-UCLAF Company, by the Ford Foundation (grant 77-0312) and the Belgian FRSM (grant 3.4514.75).

Received 29 October 1979; accepted 14 January 1980.

- Weddington, S. C. *et al.* *Nature* **254**, 145-146 (1975).
- Hansson, V. *et al.* *Nature* **250**, 387-391 (1974).
- Musto, N. A. & Bardin, C. W. *Steroids* **28**, 1-11 (1976).
- Fakunding, J. L., Tindall, D. J., Dedman, J. R., Mena, C. R. & Means, A. R. *Endocrinology* **98**, 392-402 (1976).
- Danzo, B. J., Cooper, T. G. & Orgebin-Crist, M. C. *Biol. Reprod.* **17**, 64-77 (1977).
- Jegou, B., Dacheux, J. L., Terqui, M., Garnier, D. H. & Courot, M. *Molec. cell. Endocr.* **9**, 335-346 (1978).

- Ritzen, E. M., Nayfeh, S. N., French, F. S. & Dobbins, M. C. *Endocrinology* **89**, 143-151 (1971).
- Mickelson, K. E. & Petra, P. H. *J. biol. Chem.* **253**, 5293-5298 (1978).
- Renoir, J. M., Fox, L. L., Baulieu, E. E. & Mercier-Bodard, C. *FEBS Lett.* **75**, 83-88 (1977).
- Gunsalus, G. L., Musto, N. A. & Bardin, C. W. *Science* **200**, 65-66 (1978).
- Kirchhoff, J., Soffié, M. & Rousseau, G. G. *J. Steroid Biochem.* **10**, 487-497 (1979).
- Cunningham, G. R., Tindall, D. J. & Means, A. R. *Steroids* **33**, 261-276 (1979).
- Devis, R. & Bui, X. H. *Bull. Soc. chim. Fr.* **11-12** (II), 1-8 (1979).
- Bonne, C. & Raynaud, J. P. *Steroids* **26**, 227-232 (1975).
- Rousseau, G. G. & Schmit, J. P. *J. Steroid Biochem.* **8**, 911-919 (1977).
- Hansson, V., Ritzen, M. E., French, F. S., Weddington, S. C. & Nayfeh, S. N. *Molec. cell. Endocr.* **3**, 1-20 (1975).
- Vigersky, R. A., Loriaux, D. L., Howards, S. S., Hodgen, G. B., Lipsett, M. B. & Chrambach, A. *J. clin. Invest.* **58**, 1061-1068 (1976).
- Lipschultz, L. I., Tsai, Y. H., Sanborn, B. M. & Steinberger, E. *Fertility Sterility* **28**, 947-951 (1977).
- Hsu, A. F. & Troen, P. *J. clin. Invest.* **61**, 1611-1619 (1978).
- Purvis, K., Calandra, R., Sander, S. & Hansson, V. *Int. J. Andrology* **1**, 531-548 (1978).
- Tindall, D. J., Cunningham, G. R. & Means, A. R. *Int. J. Andrology Suppl.* **2**, 434-448 (1978).

High-affinity binding of ¹²⁵I-labelled mouse interferon to a specific cell surface receptor

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Previous research suggests that interferon binds to the cell surface¹, possibly by attachment to gangliosides^{2,3}. A two-component receptor system consisting of binding and activation sites has been proposed⁴. This hypothesis is supported by the finding that interferon inhibits binding of cholera toxin and thyrotropin to their receptors^{5,6} suggesting possible common receptor sites. Moreover, an antiserum against cell surface components of interferon-sensitive cells has been shown to inhibit the action of interferon⁷. However, to understand the interaction of interferon with the cell surface requires direct ligand-binding studies. I present here direct evidence that high-affinity binding of interferon to a specific cell surface receptor is an initial step in interferon action using biologically active purified ¹²⁵I-labelled mouse interferon. Labelled interferon binds specifically to interferon-sensitive mouse leukaemia L1210 cells, whereas binding to interferon-resistant L1210 cells is nonspecific. Furthermore, specific binding to monolayer cultures of mouse L929 cells is compared with nonspecific binding to chick embryo fibroblasts insensitive to the action of mouse interferon⁸.

Partially purified mouse C243 cell interferon⁹ was further purified by affinity chromatography on a sheep-anti-mouse interferon antibody agarose column as previously described^{10,11}, followed by ion exchange chromatography on CM-Sepharose CL-6B (Pharmacia). This procedure yielded highly purified interferon with a specific activity of 5×10^8 - 10^9 mouse interferon reference units per mg of protein with a recovery of 50-75% (Fig. 1a). Interferon was assayed on mouse L929 cells challenged with vesicular stomatitis virus as described previously¹⁴; protein concentrations were estimated according to the method of Lowry.

Purified interferon was iodinated with ¹²⁵I to a specific radioactivity of 70-80 μ Ci per μ g of protein using a modified lactoperoxidase-catalysed reaction¹⁵; monoiodination of a protein with a molecular weight (MW) of 35,000 should result in a specific activity of 57 μ Ci per μ g of protein. Labelled interferon was reduced with 1% 2-mercaptoethanol in 0.1% SDS; this procedure allowed recovery of 25-50% of the initial biological activity, whereas the recovery was less than 20% before reduction, in agreement with similar previously reported results (ref. 16, Fig. 1b, c). Control experiments performed with cold iodine,

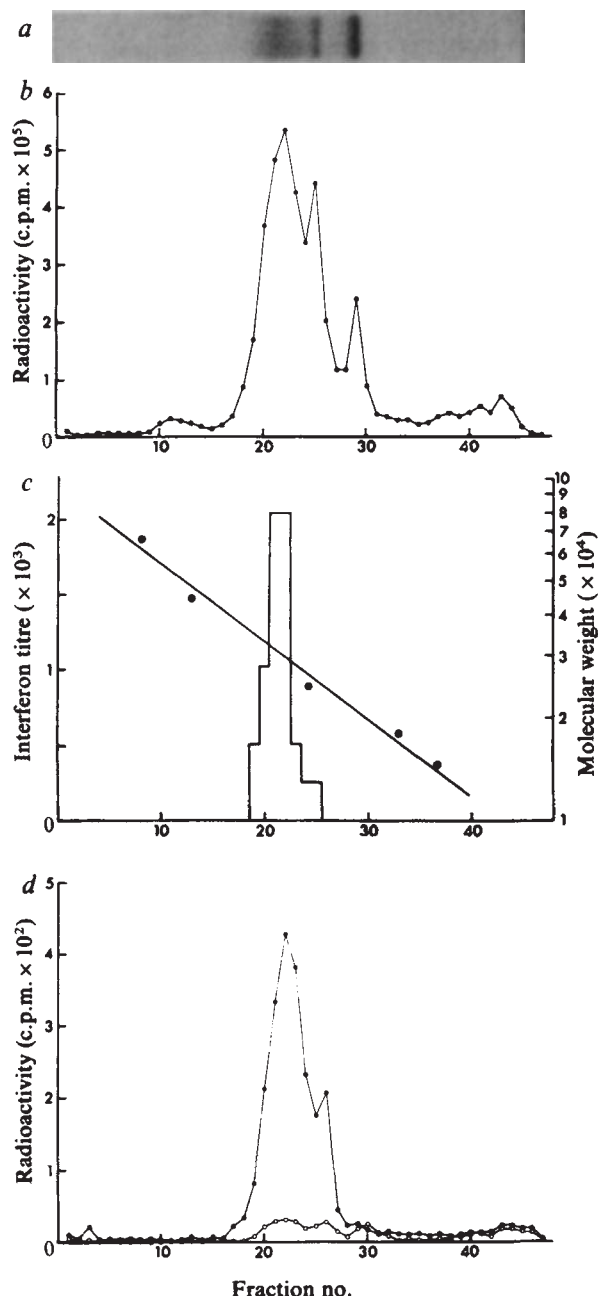


Fig. 1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli¹². Homogeneous slab gels of 12.5% acrylamide, 0.8 mm thick, 90 mm long and 130 mm wide were overlaid with a 10 mm long 3% stacking gel and run with a constant current of 10 mA in presence of 0.1% SDS (BDH). The samples were dialysed against 0.125 M Tris-HCl (pH 6.8) containing 1% SDS and heated for 2 min in a boiling water bath after addition of 1% 2-mercaptoethanol (Eastman). The sample size was 25 μ l. *a*, Coomassie blue staining of purified interferon. After elution from 2 mm gel slices, antiviral activity was associated with all three bands which correspond to apparent MWs of 35,000, 28,000 and 22,000. This interferon therefore appears to be electrophoretically similar to a recently described purified interferon prepared from Newcastle-disease-virus infected mouse Ehrlich ascites tumour cells¹³. *b*, Profile of radioactivity of 125 I-labelled purified interferon. The fractions correspond to gel slices of 2 mm. *c*, Profile of eluted antiviral activity from the material depicted in *b*. The profile is congruent with the radioactivity peaks at MWs of 35,000 and 28,000; however no antiviral activity was detectable at MW 22,000. Molecular weight markers (Sigma) were bovine serum albumin (66,000), egg albumin (45,000), trypsinogen (24,000), beta-lactoglobulin (18,400) and lysozyme (14,300). *d*, Profile of radioactivity of bound and eluted 125 I-interferon from L1210-S (●) and L1210-R (○) cells.

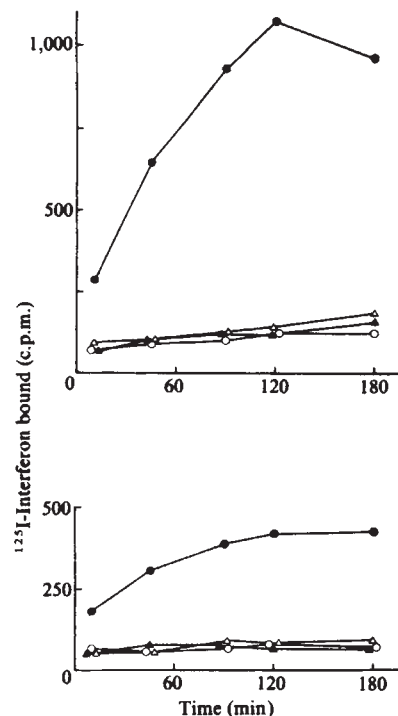


Fig. 2 The continuous cell lines of L1210-S and L1210-R cells were grown in non-agitated suspension cultures in RPMI 1640 medium (Flow laboratories) supplemented with 10% fetal calf serum (Gibco Bio-Cult)¹⁹. Cells (2×10^6) were incubated with 125 I-interferon in 24-well Costar 3524 plates at a density of 2×10^6 cells per ml and subsequently washed three times with culture medium by centrifugation at 4 °C. All experiments were performed in triplicate. The radioactivity was measured in a Gammatic gamma counter. *a*, Time-course of 125 I-interferon binding to L1210-S (●) and L1210-R (○) cells at a concentration of 400 U ml^{-1} at 37 °C. Simultaneous addition of 4×10^5 U ml^{-1} of unlabelled interferon inhibits binding to L1210-S (▲) cells, whereas binding to L1210-R (△) cells is unaltered; *b*, time course of binding at 4 °C. Standard deviations were less than 5%.

or omitting iodine in otherwise identical labelling conditions, suggest that the irreversible inactivation on iodination is due either to conformational changes as a consequence of substitution or to iodine-induced irreversible oxidation reactions.

Mouse leukaemia L1210 cells have been characterised extensively in terms of sensitivity to the various biological effects of interferon^{17,18}. An interferon-resistant subline of these cells has been cloned and the resistance to the different effects of interferon is well documented¹⁹. The time-course of 125 I-interferon binding to both cell lines is shown in Fig. 2: binding to interferon-sensitive L1210 cells (L1210-S) occurred rapidly and progressively and could be competitively inhibited by simultaneous addition of an excess of unlabelled interferon. Binding to interferon-resistant L1210 cells (L1210-R) was minimal and independent of the addition of unlabelled ligand. This binding can therefore be referred to as nonspecific. These findings confirm previous experiments on the recovery of antiviral activity from L1210-S cells, but not from L1210-R cells on treatment with large amounts of interferon¹⁹. While maximum specific binding was achieved after 120 min independently of temperature, the amount of specifically bound material was markedly higher at 37 °C than at 4 °C. We are now investigating whether this increased binding at 37 °C reflects a continuous uptake of interferon, as has been suggested previously²⁰.

The saturation of specific receptor sites for interferon is depicted in Fig. 3. Binding of 125 I-interferon to L1210-R cells as

a function of the concentration of added labelled ligand was linear and thus nonspecific; no saturation occurred in the range of biologically active concentrations. The specific receptor sites on L1210-S cells, however, were saturated between interferon concentrations of 3,200 and 6,400 units per ml, at 37 °C as well as at 4 °C (data not shown). Thus the saturation curve is congruent with the biological dose-response curve reported previously²¹.

To characterise this bound fraction further, L1210-S cells were incubated for 120 min with 6,400 units per ml of labelled interferon in the cold, washed and subsequently incubated with a 2,000-fold excess of unlabelled interferon for another 2 h at 4 °C. In these conditions 70% of the bound radioactive material was recovered, compared to less than 25% spontaneously released material. On analysis by SDS-PAGE the radioactivity profile of the specifically bound and eluted material corresponds to the profile of antiviral activity of the original ¹²⁵I-interferon preparation (Fig. 1c, d). Control experiments in which ¹²⁵I-interferon was bound and eluted from L1210-R cells confirmed the nonspecific binding to these cells (Fig. 1d).

The comparison of binding of ¹²⁵I-labelled mouse interferon to interferon-sensitive mouse monolayer L929 cells with binding to chick embryo fibroblasts, insensitive to mouse interferon⁸, yielded similar results to those obtained with L1210-S and L1210-R cells (data not shown). In contrast to the specific binding to mouse L929 cells binding of ¹²⁵I-labelled mouse interferon to chick embryo fibroblasts could not be inhibited competitively by simultaneous addition of an excess of unlabelled mouse interferon. Specific binding to mouse L929 cells revealed saturation at a concentration of 6,400 units per ml interferon and can thus be expected to occur with a similar high affinity as on L1210-S cells; however, chick embryo fibroblasts could not be saturated with ¹²⁵I-labelled mouse interferon in the same range of concentrations. Binding of mouse interferon to chick embryo fibroblasts could thus be characterised as nonspecific, in agreement with the observation that interferon can be recovered from homologous, but not from heterologous cells previously treated with high amounts of interferon²².

The various approaches for the characterisation of the specific interaction between high affinity membrane receptors and their ligands has been the subject of reviews by Kahn²³ and Cuatrecasas²⁴. The mass action law can serve as a model for the interaction of a ligand with its receptor on the assumption that the binding corresponds to a simple bimolecular equilibrium. It can be derived from the equation of mass action law, that the dissociation constant at half saturation of the receptor sites is equal to the concentration of free ligand at half saturation. As less than 10% of the added labelled interferon was bound to the cells at half saturation, the concentration of free ligand can be set equal to the known total ligand concentration. Assuming that labelled interferon possesses identical binding properties as the unlabelled material and that only biologically active labelled interferon binds specifically, an apparent affinity constant for binding of interferon to L1210-S cells can be derived from the saturation curve depicted in Fig. 3: half saturation is achieved at a concentration of 400–800 units per ml, equivalent to $1\text{--}2 \times 10^{-11}$ M for a mean MW of 35,000 and a specific activity of 10^9 units per mg protein.

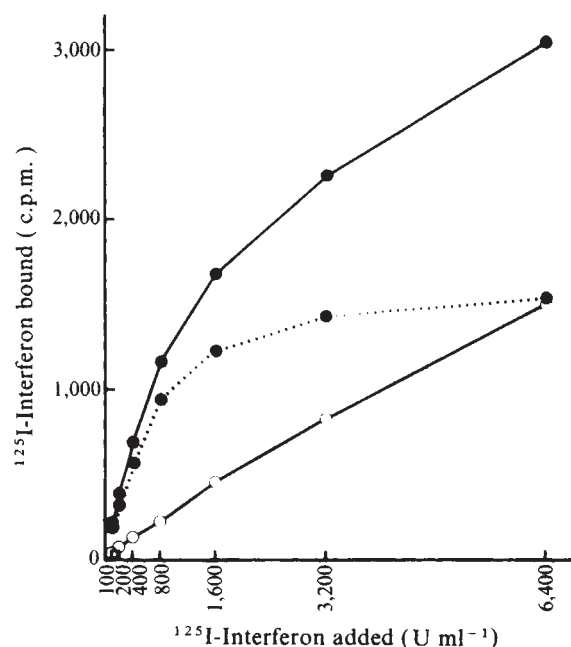


Fig. 3 Saturation of ¹²⁵I-interferon binding to L1210-S (●) and L1210-R (○) cells after 2 h incubation at 37 °C. The specific binding is depicted as the difference between total binding to L1210-S cells and nonspecific binding to L1210-R cells (dotted line). Standard deviations were less than 5%.

The present results provide, for the first time, direct evidence for high affinity binding to a specific cell surface receptor as the initial step in interferon action. Thus interferon binding shares a characteristic property of other biologically highly active substances such as peptide hormones and neurotransmitters²³. This strongly suggests that the mechanism of resistance to interferon across the barrier of species specificity, as well as the resistance of L1210-R cells is located at the cell surface. Ligand binding studies offer new possibilities for a direct approach to the study of several aspects of interferon action. Thus interferon antagonists, interferon derivatives or heterologous interferons may be analysed directly with regard to competitive binding and biological crossreactivity. Furthermore, use of biologically active, purified ¹²⁵I-interferon offers new possibilities to investigate binding and pharmacokinetic properties *in vivo*.

I thank Dr I. Gresser for encouragement and help, as well as for reviewing the manuscript, Dr R. Monier for his interest and suggestions, Drs M. Tovey and E. Mogensen for helpful discussions and Mrs B. Blanchard for technical assistance. This work was partly supported by grants from DRET (78-34-210), DGRST (79-7-0205) and INSERM (ATP 78-90). M.A. was supported by a Postdoctoral Fellowship from the Swiss NSF.

Received 28 November 1979; accepted 8 February 1980.

- Friedman, R. M. *Science* **156**, 1760–1761 (1967).
- Besançon, F. & Ankel, H. *Nature* **252**, 478–480 (1974).
- Vengris, V. E., Reynolds, F. H. Jr, Hollenberg, M. D. & Pitha, P. M. *Virology* **72**, 486–493 (1976).
- Chany, C. *Biomedicine* **24**, 148–157 (1976).
- Kohn, L. D., Friedman, R. M., Holmes, J. M. & Lee, G. *Proc. natn. Acad. Sci. U.S.A.* **73**, 3695–3699 (1976).
- Grollman, E. F. *et al. Cancer Res.* **38**, 4172–4185 (1978).
- Revel, M., Bash, D. & Ruddell, F. H. *Nature* **260**, 139 (1976).
- Baron, S., Barban, S. & Buckler, C. E. *Science* **145**, 814–815 (1964).
- Tovey, M. G., Begon-Lours, J. & Gresser, I. *Proc. Soc. exp. Biol. Med.* **146**, 809–815 (1974).
- Sipe, J. D., De Maeyer-Guignard, J., Fauconnier, B. & De Maeyer, E. *Proc. natn. Acad. Sci. U.S.A.* **70**, 1037–1040 (1973).
- De Maeyer-Guignard, J., Tovey, M. G., Gresser, I. & De Maeyer, E. *Nature* **271**, 622–625 (1978).

- Laemmli, U. K. *Nature* **227**, 680–685 (1970).
- Carber, B. *et al. J. Biol. Chem.* **254**, 3681–3684 (1979).
- Gresser, I., Fontaine-Brouty-Boydé, D., Bourali, C. & Thomas, M.-T. *Proc. Soc. exp. Biol. Med.* **130**, 236–241 (1969).
- David, G. S. & Reisfeld, R. A. *Biochemistry* **13**, 1014–1021 (1974).
- Stewart II, W. E., De Somer, P. & De Clercq, E. *J. gen. Virol.* **24**, 567–570 (1974).
- Gresser, I., Brouty-Boydé, D., Thomas, M.-T. & Macieira-Coelho, A. *Proc. natn. Acad. Sci. U.S.A.* **66**, 1052–1058 (1970); *J. natn. Cancer Inst.* **45**, 1145–1153 (1970).
- Gresser, I., Bandu, M.-T., Tovey, M. G., Bodo, G., Paucker, K. & Stewart II, W. E. *Proc. Soc. exp. Biol. Med.* **142**, 7–10 (1973).
- Gresser, I., Bandu, M.-T. & Brouty-Boydé, D. *J. natn. Cancer Inst.* **52**, 553–559 (1974).
- Stewart II, W. E., in *Effects of Interferon on Cells, Viruses and the Immune System* (ed. Gherlache, A.) 75–98 (Academic, New York, 1975).
- Gresser, I. & Tovey, M. G. *Biochim. biophys. Acta* **516**, 231–247 (1978).
- Stewart II, W. E., De Clercq, E. & De Somer, P. *J. Virol.* **10**, 707–712 (1972).
- Kahn, C. R. *J. Cell Biol.* **70**, 261–286 (1976).
- Cuatrecasas, P. & Hollenberg, M. D. *Adv. Protein Chem.* **30**, 251–451 (1976).

Protein kinase in immunoprecipitate of DMBA-transformed epithelial cells with serum from tumour-bearing rabbits

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Recent work using virally transformed cells has shown that the viral gene product responsible for transformation is associated with protein kinase activity^{1,2}. Use of antisera specific for the *src* gene product of Rous sarcoma virus showed that the heavy chain of the antibody in the immunoprecipitate can be phosphorylated by the bound antigen *src* gene product. Only a very low level of such protein kinase activity was observed using uninfected cells or cells infected with transformation-defective virus. Such a low level of activity has been attributed to the endogenous *src* gene product^{3,4}. Similar protein kinase activity, capable of phosphorylating IgG, was also detected in the immunoprecipitate of the T antigen of adenovirus type 5 (ref. 5). Moreover, large T antigen of SV40 is also associated with protein kinase and/or ATPase activity^{6,7}. Thus, many of the virally coded proteins involved in transformation are apparently associated with protein kinase activity. In addition, secretion of specific phosphoproteins and increased protein kinase activity have been detected in conditioned medium from cells transformed by oncogenic viruses⁸. Secretion of these proteins, accompanied by an additional protein kinase, correlates well with the transformation as demonstrated by the use of temperature-sensitive mutants in the *src* gene of avian sarcoma virus. These data suggest that the alteration in protein phosphorylation may contribute to the transformed state. In the virion of murine sarcoma virus but not murine leukaemia virus, a novel protein kinase with molecular weight of 15,000 (15 K) has recently been discovered⁹. In view of these findings, it is of interest to investigate whether in chemical carcinogen transformed epithelial cell an analogous event also occurs. We studied transformed bladder epithelial cells induced after *in vitro* exposure to the chemical carcinogen, 7,12-dimethylbenz[*a*]anthracene (DMBA). Anti-serum against DMBA-transformed mouse bladder epithelial cells were raised in rabbits and used to immunoprecipitate antigens from chemically transformed cell lysates. By incubating the immune complex with [γ -³²P]ATP we found that a transformation-specific 18K protein becomes phosphorylated. Such a phosphorylatable 18K protein is also detected in the conditioned medium of DMBA-transformed cells.

Background work on the establishment, characterisation and tumorigenicity of the DMBA-transformed bladder epithelial cell lines has been published previously¹⁰. Tumours were induced by subcutaneous injection of DMBA-transformed mouse bladder epithelial cells ($1-2 \times 10^6$ cells) into 5-week old New Zealand rabbits. Three more weekly injections of the same cell line into the same site were given. One week after the last injection, blood was collected. Of the five antisera raised against different epithelial cell lines, immunoprecipitates from four gave identical results in the phosphorylation of an 18K protein. One of them, raised against MB49 and designated serum V, was used in this study because of its higher titre.

Cytoplasmic extracts prepared from cultures of primary cells, untransformed cell lines and chemically transformed epithelial cells, were immunoprecipitated with immune or non-immune serum on protein A-Sepharose beads. Extensively washed immunoprecipitates were then incubated with [γ -³²P]ATP, rewashed and analysed by SDS polyacrylamide gel electrophoresis. Figure 1e shows an immunoprecipitate of a DMBA-transformed epithelial cell line with phosphorylation of an 18K

protein which could sometimes be resolved as a doublet. Similar results were observed when the immunoprecipitate was made to a benzo[*a*]pyrene (BP) transformed rabbit bladder epithelial cell line. Non-immune serum (Fig. 1c,f) and a variety of other antisera against fibronectin, p60^{src}, FOCMA, SV40-T antigen and polyoma-T antigen, did not support a phosphotransfer reaction in the immunoprecipitate of the DMBA-transformed epithelial cells. We also failed to detect ³²PO₄-labelled 18K proteins in immunoprecipitates of immune serum V reacted with primary cultures of epithelial cells, fibroblasts and endothelial cells (Fig. 1h, i, j respectively). However, among a variety of established cell lines tested, the phosphorylation of the 18K protein was detected in immunoprecipitates of mouse BALB 3T3-A31 cells and CCL146 (gerbil fibroma) cells with immune serum V (Fig. 1b). In other established cell lines (Rat 1 and NRK) scored negative for protein kinase (Table 1), small amounts of radioactive incorporation in the 18K region could be detected (Fig. 1g) after prolonged exposure of the gel. Cell lines which failed to react with serum V to give the 18K protein included RK13 (rabbit kidney), CCL60 (rabbit fibroblast), CV-1 (monkey kidney) and RPL (rat liver). For virally transformed cells tested, no detectable phosphorylatable 18K was observed in immunoprecipitates with immune serum V in these experimental conditions. These include RSV-chick, RSV-rat (CCL47), SV80 (SV40 human cells), 64F3 (FeSV-CCL64) and T8 (adenovirus types 2 transformed rat cells). It should also be noted that the incubation of [γ -³²P]ATP with the immunoprecipitate of the *src* gene product of RSV or the T antigen of SV40 or polyoma virus does not result in the appearance of ³²P-labelled 18K protein.

When immune serum V was incubated with formaldehyde-fixed DMBA-transformed epithelial cells (MB49), and then

Table 1 Protein kinase activity in immunoprecipitates of different cell types with tumour-bearing serum

Cell types		Relative amount of ³² PO ₄ -labelled 18K protein, % of MB49
<i>Primary cultures</i>		
(1) Embryo fibroblasts	rat	0
(2) Embryo fibroblasts	chick	0
(3) Embryo fibroblasts	Chinese hamster	0
(4) Bladder epithelium	mouse	0
(5) Bladder epithelium	rabbit	0
(6) Aorta endothelium	cow	0
<i>Spontaneous cell lines</i>		
(1) CV-1	Monkey	0
(2) NRK	rat	5%
(3) Rat 1	rat	2%
(4) RPL	rat	0
(5) RK13	rabbit	0
(6) CCL 60	rabbit	0
(7) BALB 3T3-A31	mouse	80%
<i>Chemically transformed epithelial cell lines</i>		
(1) MB33 I	mouse-DMBA	100%
(2) MB33 II	mouse-DMBA	100%
(3) MB48 B	mouse-DMBA	100%
(4) MB49	mouse-DMBA	100%
(5) MB49 B	mouse-DMBA	100%
(6) MB63	mouse-DMBA	100%
(7) MB71 B	mouse-DMBA	100%
(8) RBC	rabbit-benzo[<i>a</i>]pyrene	100%
<i>Tumour-derived cell lines</i>		
(1) CCL146	gerbil-fibroma	100%
(2) B103	rat-neuronal tumour	0
<i>Virally transformed cell lines</i>		
(1) CCL47	rat-RSV	0
(2) 64F3	mink-FeSV	0
(3) SV80	human-SV40	0
(4) CEF/Ts68	chick-RSV	0
(5) T8	rat-adenovirus 2	0

used to immunoprecipitate antigens for chemically transformed cell lysates, phosphorylation of the 18K protein was greatly reduced (Fig. 1a, d). Removal of such activity can also be accomplished by incubation of antiserum with live DMBA-transformed cells. However, absorption of tumour-bearing serum V on fixed primary epithelial cells or cells scored negative for the phosphorylated 18K protein does not result in the reduction of phosphorylation activity after immunoprecipitation. Thus, it is reasonable to conclude that the phosphorylation activity observed in immunoprecipitate is induced after oncogenic transformation by the chemical carcinogen.

Antiserum V, although raised in the rabbit by injecting mouse cells, is not species specific. It recognises an 18K protein in mouse, rat, rabbit and gerbil cell lines. As summarised in Table 1, this 18K protein seems to be transformation-dependent. As the ^{32}P -labelled 18K protein is not detectable in immunoprecipitates of serum V with a variety of virally transformed cells, this protein is probably not a common transformation-dependent protein. Rather, it seems to be confined to only chemically transformed cells and a few immortal cell lines.

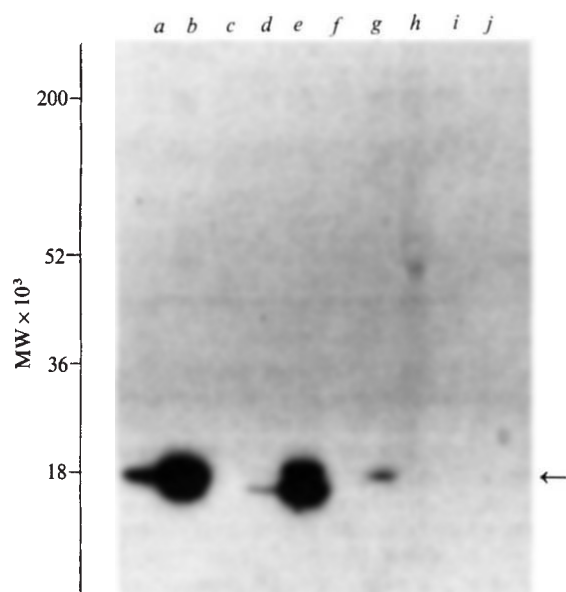


Fig. 1 Detection of a protein kinase activity in immunoprecipitates of various cell types. Cytoplasmic extracts were prepared and immunoprecipitated with tumour-bearing rabbit serum V, serum V absorbed with formaldehyde fixed DMBA-transformed mouse epithelial cells, MB49, or non-immune serum. Cells (2×10^6) were washed five times with STE buffer (0.15 M NaCl/0.1 M Tris-HCl, pH 7.2/0.001 M EDTA) and lysed in 0.7 ml of RIPA buffer (1.0% Triton X-100/1.0% sodium deoxycholate/0.1% SDS/0.15 M NaCl/0.01 M Tris-HCl, pH 7.2) supplemented with 200 units ml^{-1} of Trasylol (Calbiochem) at 4 °C. Cell lysate (0.5 ml) was mixed with 10 μl of non-immune serum and 40 μl of protein A Sepharose CL-4B (Pharmacia). After 10 min of gentle agitation the mixture was centrifuged at 28,000g for 30 min and the supernatant was removed for immunoprecipitation. To 100 μl aliquots of supernatant 10 μl of immune serum 40 μl of protein A-Sepharose was added and incubated at 4 °C for 30 min with constant mixing. The immune complexes were washed four times with RIPA buffer and then once with phosphate-buffered saline (PBS). The pellet was incubated at room temperature for 20 min in a buffer containing 0.1 M Tris, pH 7.4; 1 mM MgCl_2 and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (0.1 μM , specific activity 2000 Ci mmol^{-1} ; NEN) and then washed four times with PBS to remove unbound radioactivity. The immune complexes were eluted from the Sepharose beads by boiling in buffer containing 1% SDS and 2% β -mercaptoethanol for 4 min. The supernatant was then analysed by SDS polyacrylamide (12.5%) slab gel. The gel was stained with Coomassie brilliant blue, dried and an autoradiograph was prepared by Kodak SB-5 film. Cell line derived from gerbil benign tumour, CCL146 with a, absorbed serum V; b, serum V; c, non-immune serum. DMBA-transformed mouse bladder epithelial cell line MB49 with d, absorbed serum V; e, serum V; f, non-immune serum; g, NRK with serum V. Primary cultures with serum V, h, mouse bladder epithelium; i, hamster embryo fibroblasts; j, bovine endothelial cells. The molecular weight markers used were myosin heavy chain (200K), tubulin (52K), troponin T (36K) and lactoglobulin (18K).

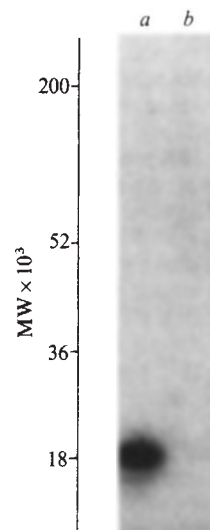


Fig. 2 Protein kinase in immunoprecipitates of conditioned medium from normal and chemically transformed cells. Conditioned medium immunoprecipitated with serum V, was incubated for 20 min with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ at room temperature. Immune complexes were eluted from Sepharose beads and analysed on an SDS polyacrylamide slab gel. Conditioned medium from a, DMBA-transformed epithelial cell line, MB49; b, primary culture of mouse bladder epithelium. The molecular weight markers used are myosin heavy chain (200K), tubulin (52K), troponin T (36K) and lactoglobulin (18K).

Serum-free medium was conditioned for 24 h with MB49 cells, collected from the dish and centrifuged to clear it of any cells. The conditioned medium was either incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 20 min and then immunoprecipitated with serum V or immunoprecipitated first and then incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Both procedures resulted in the detection of ^{32}P -labelled 18K protein from DMBA-transformed cells (Fig. 2a) but not primary epithelial cells (Fig. 2b). The phosphorylation of an 18K protein in conditioned medium was destroyed when conditioned medium was first heated to 100 °C for 1 min before incubation with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ but was unaffected by heat treatment at 56 °C for 30 min.

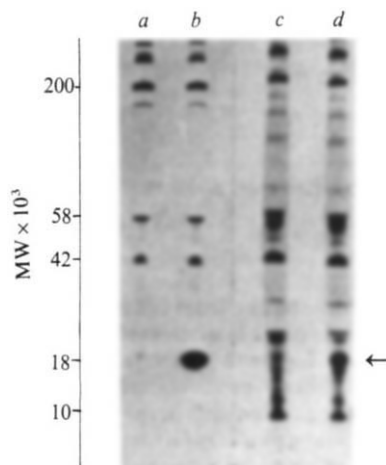


Fig. 3 SDS polyacrylamide gel analysis of immunoprecipitate of ^{35}S -methionine or ^{32}P -phosphate labelled conditioned medium from DMBA-transformed epithelial cell line, MB49, with tumour-bearing serum V. Confluent cells were incubated with ^{35}S -methionine (NEN, 400 Ci mmol^{-1} , 75 $\mu\text{Ci ml}^{-1}$) or ^{32}P -phosphate (NEN, 2,000 Ci mmol^{-1} , 200 $\mu\text{Ci ml}^{-1}$) in Dulbecco-modified Eagle's medium deficient in methionine or phosphate for 24 h. After centrifugation at 100g for 10 min, the supernatant was used for immunoprecipitation a, ^{35}S -methionine labelled, non-immune serum; b, ^{35}S -methionine labelled, tumour-bearing serum; c, ^{32}P -phosphate labelled, non-immune serum; d, ^{32}P -phosphate labelled, tumour-bearing serum. The molecular weight markers used were myosin heavy chain (200K), vimentin (58K), egg albumin (42K) and lactoglobulin (18K).

Evidence that the 18K protein is synthesised by cells and not acquired from serum was demonstrated by the incorporation of ^{35}S -methionine and ^{32}P -phosphate into 18K protein (Fig. 3) and the fact that in the absence of serum, cells continued to synthesise this protein and shed it into the culture medium.

Phosphorylation of the 18K protein, in immunoprecipitates of cell lysate or conditioned medium, was neither cyclic AMP nor cyclic GMP dependent. The reaction required a proper concentration of Mg^{2+} (1 mM) and was not inhibited by Ca^{2+} (1–10 mM). Intriguingly, it was inhibited by F-actin, similar to that of protein kinase associated with the *src* gene product¹¹ or with murine sarcoma virus⁹. Both ATP and GTP can be utilised for the phosphotransfer reaction. In many respects, the profile of phosphorylation of the 18K protein is similar to the phosphorylation of IgG heavy chain in the immunoprecipitate of *src* gene product.

The presence of a transformation-associated protein kinase activity reported in this study is similar, conceptually, to that reported for virally transformed cells^{1,2} but differs with respect to the substrate phosphorylated in similar experimental conditions. Serum directed against p60^{src}, the product of the *src* gene

of avian sarcoma virus, does not immunoprecipitate any detectable kinase activity from DMBA-transformed cells and conversely, immunoprecipitates of serum V with virally transformed cells does not result in the phosphorylation of the heavy chain of IgG on addition of [γ - ^{32}P]ATP. Since alteration of protein phosphorylation plays an important part in several biological processes^{12–14}, the difference found between normal and chemically transformed cells in this respect may be of significance to the understanding of transformation induced by chemical carcinogens.

The possibility that our transformation-associated phosphoprotein may have growth-promoting properties¹⁵, protease activity¹⁶ or angiogenesis activity¹⁷ are currently under investigation. The isolation of the 18K phosphoprotein should help identify its biochemical role and clarify as to whether the expression and secretion of this protein is a causative or secondary event in transformation induced by chemical carcinogens such as DMBA or BP.

We thank Beth Shepherd and Rebecca Reynold for technical assistance. This work was supported by a grant from National Cancer Institute (P01CA22427-02).

Received 8 August 1979; accepted 15 February 1980.

- Collett, M. S. & Erikson, R. L. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2021–2024 (1978).
- Levinson, A. D., Opperman, H., Levintow, L., Varmus, H. E. & Bishop, J. M. *Cell* **15**, 561–572 (1978).
- Collett, M. S., Erikson, E., Purchio, A. F., Brugge, J. S. & Erikson, R. L. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3159–3163 (1979).
- Opperman, H., Levinson, A. D., Varmus, H. E., Levintow, L. & Bishop, J. M. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1804–1808 (1979).
- Lassam, N. J., Bayley, S. T., Graham, F. L. & Branton, P. E. *Nature* **277**, 241–243 (1979).
- Tjian, R. & Robbins, A. *Proc. natn. Acad. Sci. U.S.A.* **76**, 610–614 (1979).
- Griffin, J. D., Spangler, G. & Livingston, D. M. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2610–2614 (1979).
- Senger, D. R., Wirth, D. F. & Hynes, R. O. *Cell* **16**, 885–893 (1979).
- Sen, A. & Todaro, G. J. *Cell* **17**, 347–356 (1979).
- Summerhayes, I. C. & Franks, L. M. *J. nam. Cancer. Inst.* **62**, 1017–1023 (1979).
- Tsen, S. D., Lee, R., Damiani, B., Hsieh, P. & Chen, L. B. *Cold Spring Harb. Symp. quant. Biol.* **44** (in the press).
- Rubin, C. S. & Rosen, O. M. A. *Rev. Biochem.* **44**, 831–887 (1975).
- Langan, T. A. *Adv. Cyclic Nucleotide Res.* **3**, 99–153 (1973).
- Greengard, P. *Science* **199**, 146–152 (1978).
- DeLarco, J. E. & Todaro, G. J. *Proc. natn. Acad. Sci. U.S.A.* **75**, 4001–4005 (1978).
- Unkles, J. C. *et al. J. exp. Med.* **137**, 85–111 (1973).
- Folkman, J., Hochberg, M. & Knighton, D. in *Control of Proliferation in Animal Cells* (eds Clarkson, B. & Baserga, R.) (Cold Spring Harbor Laboratory, New York, 1974).

Characterisation of an adrenal zona glomerulosa-stimulating component of posterior pituitary extracts as α -MSH

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The secretion of aldosterone from the zona glomerulosa of the mammalian adrenal cortex is stimulated by ACTH, potassium, angiotensin II and III, growth hormone, serotonin and E series prostaglandins^{1–7}. Some experimental and clinical studies suggest that additional stimulants of the zona glomerulosa must exist, possibly including pituitary factors other than ACTH^{6,8–13}. The possibility that posterior pituitary extracts may contain a zona glomerulosa stimulant was first suggested 20 years ago^{14,15}, but has since received little attention. We describe here the purification from posterior pituitary extracts of activities that stimulate rat glomerulosa cells and whole tissue *in vitro*. One of the active compounds has been identified as α -MSH (melanocyte stimulating hormone).

Two posterior pituitary extracts were investigated, one an extract of bovine posterior pituitaries¹⁶, the other Pitressin (Parke Davis). Their effects on the production of corticosterone by adrenal cell suspensions compared to ACTH are shown in Table 1. Values obtained for the stimulation by ACTH are

consistent with those obtained previously in these laboratories, and also with those reported by other workers^{1,17,18}. Posterior pituitary extract 1 also stimulated both glomerulosa and inner zone adrenal cells, but the dose required to give half-maximal stimulation is lower than expected on the basis of immunoassayable ACTH by a factor of about 17 for glomerulosa cells based on the C-terminal assay¹⁹, but only 6.6 for the N-terminal assay¹⁹, and of about 10 and 3 respectively for fasciculata/reticularis cells. Pitressin stimulates glomerulosa cells alone and has no effect on inner zone cells. Neither vasopressin, substance P nor neurotensin have any effect on either cell type in the conditions used.

For bioassay purposes, the production of corticosterone alone was used as a measure of steroidogenesis. However, the effect of the stimulants on the total steroid profile of the glomerulosa using whole tissue preparations is shown in Fig. 1. It can be seen

Table 1 Potencies of various stimulants on rat adrenocortical cell suspensions

	ACTH (mol l ⁻¹)	Posterior pituitary extract no. 1 (μ g)	Pitressin (pressor units)
Glomerulosa cells	6.6×10^{-11}	13.7 (4×10^{-12} C-terminal; 4×10^{-11} N-terminal)	108 mU
Fasciculata/ reticularis cells	6.9×10^{-11}	20 (6×10^{-12} C-terminal; 1.5×10^{-11} N-terminal)	NS

Values are amounts of stimulant which in standard incubation conditions give half maximal stimulation of corticosterone production. Units are: ACTH, mol l⁻¹; posterior pituitary extract 1, μ g per flask (values in parenthesis are expected ACTH contamination as measured by two radioimmunoassays¹⁹, in mol l⁻¹); Pitressin, pressor units per flask. NS, no stimulation.

that qualitatively similar results were obtained with ACTH, posterior pituitary extract 1, and Pitressin. Corticosterone and 18-hydroxycorticosterone (18-OH-B) are most stimulated, with a smaller increment in aldosterone. The effect on deoxycorticosterone (DOC) is variable, while 18-hydroxy-DOC (18-OH-DOC) is not affected. The response of the steroid profile in intact (non-dispersed) tissue is highly characteristic and contrasts with the effects of potassium ions, or dibutyryl cyclic-AMP, both of which stimulate 18-OH-DOC as well as the other products^{20,21}.

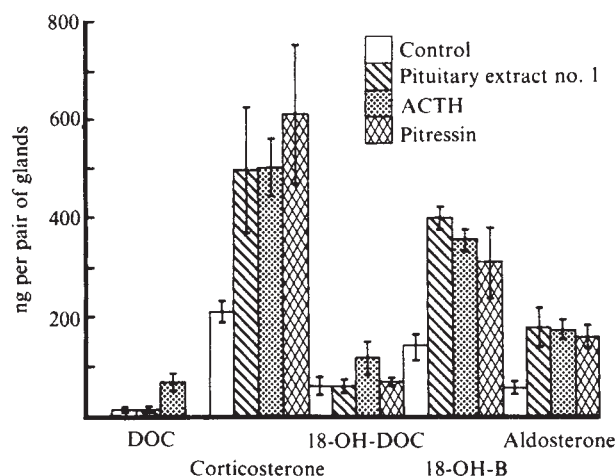


Fig. 1 Profile of steroids obtained during incubation of rat adrenal zone glomerulosa (intact) in control conditions and with the addition of ACTH ($1 \mu\text{g}$ per flask, approximately $5.5 \times 10^{-8} \text{ mol l}^{-1}$), crude posterior pituitary extract 1 ($100 \mu\text{g}$ per flask, approximately $1.5 \times 10^{-7} \text{ mol l}^{-1}$ α -MSH/ACTH as judged by the N-terminal assay) or Pitressin (2 pressor units per flask, approximately $0.6 \times 10^{-7} \text{ mol l}^{-1}$ α -MSH) to the incubation flasks. A characteristic of all these stimulants is that corticosterone, 18-OH-B and aldosterone are stimulated ($P < 0.05$ or better) whereas 18-OH-DOC is not. This contrasts with stimulation by potassium or dibutyryl cyclic AMP, which also stimulate 18-OH-DOC. Values are means $\pm$ s.e.m., $n = 6$ throughout. Male rat adrenals were used for incubation of whole tissue. Pairs of capsules which consist mostly of zona glomerulosa cells but with 5–8% contamination with fasciculata cells were incubated in individual flasks containing 5 ml Krebs–Ringer (without bovine serum albumin (BSA) or collagenase, see legend to Fig. 2). After 1 h preincubation the medium was discarded and the tissue resuspended in fresh Ringer. Incubations, to which stimulants were added, were for 2 h.

Fractionation of Pitressin was achieved using reverse-phase high pressure liquid chromatography (HPLC) in a salt-free system²² (Fig. 2, a similar profile of this preparation has been reported for a salt-based system²³). Assay for glomerulosa cell stimulation shows the activity mostly to be associated with three UV absorbing peaks indicated on the chromatogram (peaks A–C). When these are pooled and tested against unfractionated Pitressin at a range of concentrations, the dose–response curves show that at least 70% of the total glomerulosa stimulating activity in Pitressin is contained in these fractions. From sample to sample of Pitressin, however, their relative amounts vary enormously, even between vials of the same batch. In some samples the UV absorbing peaks are absent altogether, and in these the eluates from the HPLC columns are also devoid of glomerulosa-stimulating activity. Rechromatography of peak A (or B) gives a single peak in the ultraviolet co-incident with biological activity.

Peak A was further characterised in the following ways:

(1) Treatment of the active sample with leucine aminopeptidase and, separately, carboxypeptidase Y did not lead to destruction of the biological activity. However, treatment with elastase destroyed the biological activity and, overall, these data suggested the presence of a peptide blocked both at the N- and C-termini.

Table 2 Amino acid content of tubes 26 to 31 after chromatography of 1 ml of Pitressin on the preparative HPLC column (see legend to Fig. 2)

	Fraction no.					
	26	27	28	29	30	31
Asx	2.7	1.2	3.1	3.4	3.5	3.5
Thr	1.0	0.7	2.0	2.0	2.1	1.7
Ser	2.0	2.1	4.8	6.0	3.4	2.6
Glx	4.3	3.4	6.2	7.1	5.5	4.9
Pro	4.3	1.6	7.6	8.9	5.0	5.2
Gly	3.9	2.2	5.4	6.3	4.8	4.6
Ala	3.2	1.5	3.4	4.1	4.1	4.5
Val	1.7	1.2	2.8	4.3	2.8	2.2
Met	0.6	0.5	1.9	2.4	1.0	0.5
Ile	0.4	0.6	0.6	0.7	0.8	0.8
Leu	1.8	0.8	2.2	2.8	3.2	2.9
Tyr	1.4	0.9	2.3	3.0	1.7	1.0
Phe	2.0	1.4	3.0	3.8	2.3	1.6
His	1.1	1.2	2.3	3.4	1.5	1.2
Lys	1.1	1.0	2.2	2.8	1.6	1.2
Arg	1.1	1.1	2.7	3.5	1.8	1.8

Peaks A and B correspond to tubes 28 and 29 respectively, and a tentative amino acid analysis for peak A is obtained by subtraction of 'blank' tubes (26, 27, 30 or 31) from tube 28. Values are in nmol ml^{-1} .

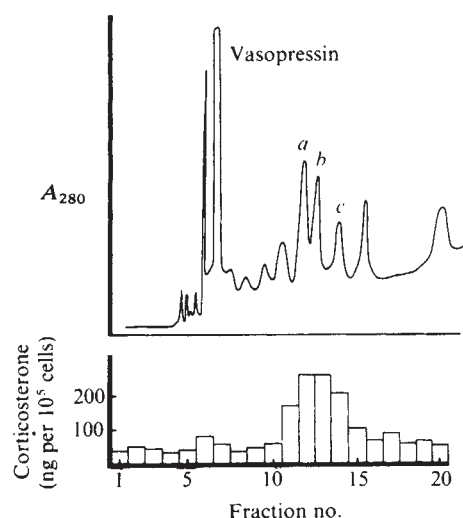


Fig. 2 UV profile of reverse-phase HPLC fractionation of Pitressin using a $0.39 \times 30 \text{ cm}$ column and the conditions given in ref. 22. A similar UV profile was obtained using a $0.78 \times 30 \text{ cm}$ column in the reported conditions²². The effect on corticosterone synthesis of adding these fractions to incubations of glomerulosa cells is shown in the lower part of the figure. For bioassays Wistar strain rats from the colony maintained at St Bartholomew's Medical College were used. Animals were killed by cervical dislocation, and the adrenals were quickly excised and stored on ice for brief periods before incubation. The glands were decapsulated and the capsules, containing largely zona glomerulosa cells (approximately 7% contamination by inner zone cells) and inner zones (zona fasciculata and zona reticularis) were incubated separately in Krebs bicarbonate Ringer solution (3.6 mM K^+) with 1% BSA (Sigma, fraction V), 200 mg glucose per 100 ml (KRBGA), and 2 mg ml^{-1} collagenase (Worthington). Tissue from 20 animals was incubated in 20 ml medium for 1 h at 37°C under 95% O_2 , 5% CO_2 . The tissue was then disrupted by repeated pipetting, filtered through nylon gauze, and centrifuged at $100g$ for 10 min. Cells were resuspended in 2 ml medium, and recentrifuged. The supernatant was discarded and the cells resuspended in a volume of incubation medium suitable for dispensing to incubation flasks.

(2) Amino acid analysis of column fractions spanning the biological activity (tubes 26–31) gave the data shown in Table 2. Subtraction of the non-active 'blanks' from the active fractions leads to a tentative conclusion that the active peptide is approximately 15 residues long with 1 Lys and 1 Arg or 30 residues long with 2 Lys and 2 Arg and so on. (3) Mass spectrometric

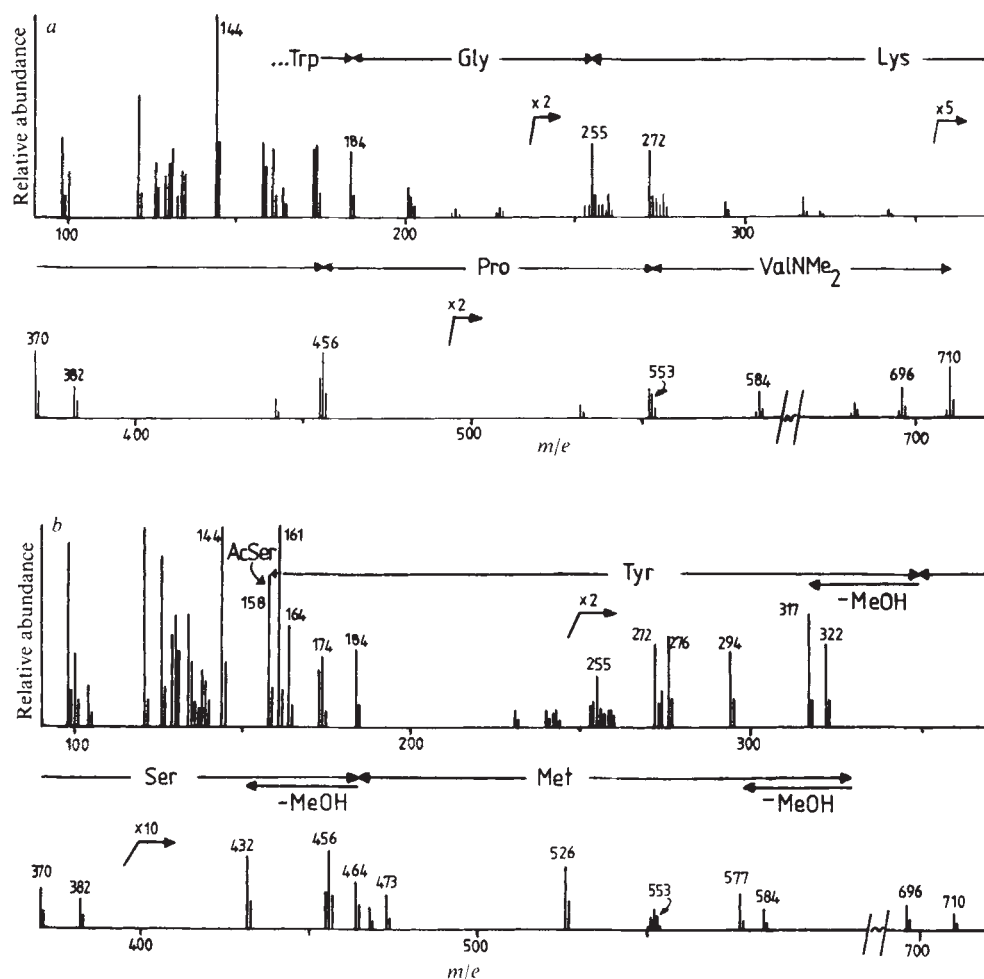
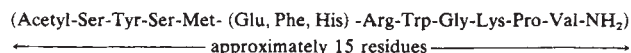


Fig. 3 Electron impact mass spectra of a tryptic digest of peak A at an ion source temperature of 300 °C (a) and at an ion source temperature of 350 °C (b). Spectra were recorded on a Kratos MS 50 mass spectrometer operating with an accelerator voltage of 8 kV and an electron beam energy of 70 eV. Before mass spectrometric examination peak A was dissolved in 100 μ l of 50 mM ammonium bicarbonate and treated with trypsin (1 μ g) for 3 h at 37 °C. After freeze drying the digest was deuterioacetylated and permethylated according to published procedures^{24,25}.

examination after trypsin digestion and derivative formation^{24,25} yielded good spectra; two representative spectra of the active material are shown in Fig. 3. The blocked N-terminus of the peptide is clearly established as acetyl-Ser-Tyr-Ser-Met- via signals at m/e 158, 317, 432, 464, 577 (Fig. 3b) and the blocked C-terminus is identified as Trp-Gly-Lys-Pro-Val-NH₂ via 'N-C cleavage' signals²⁶ at m/e 184, 255, 456, 552, 553, 696, 710 (Fig. 3a). Signals corresponding to an N-terminal Trp sequence, in addition to the N-C cleavage fragments assigned in Fig. 3, were observed in several spectra obtained during the source temperature gradient used for the examination of the tryptic digest of peak A. This suggests that the tryptophan is preceded by Lys or Arg (Arg if there is only one residue of each). Signals at m/e 98, 131, 134, 135 and 138 indicate the presence of glutamic acid, phenylalanine and histidine in the sequence of the molecule, and the lack of additional peptide signals in the spectra suggests that the peptide is likely to be approximately 15 rather than 30 residues in length. Overall the structural data obtained from the mass spectra may be summarised as



Comparison of these data with known structures of biologically active peptides suggests the probability that the active component of Pitressin is α -MSH:



which has identical N- and C-terminal sequences and whose structure is consistent with the other mass spectrometric and amino acid analysis data.

Accordingly, synthetic α -MSH was chromatographed both separately and together with Pitressin and found to co-elute with

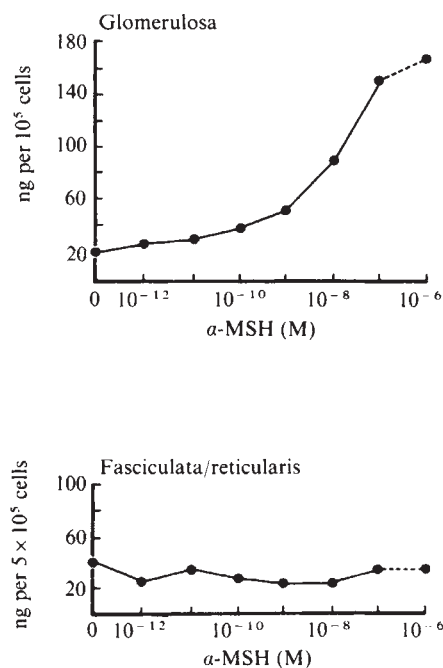


Fig. 4 Effects of α -MSH on rat adrenocortical cells (dispersed, see legend to Fig. 2) as judged by corticosterone production, at various concentrations. All values are means of two observations in a single experiment (that is, a uniform cell crop) except for dotted extrapolations which are based on data derived in a separate experiment.

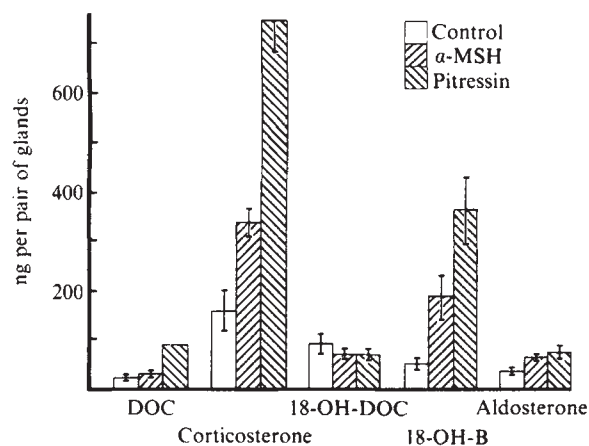


Fig. 5 Profile of steroids obtained during incubation of rat adrenal zona glomerulosa (intact tissue, see legend to Fig. 1) in control conditions and with the addition of α -MSH ($0.5 \text{ nmol per flask}$, $10^{-7} \text{ mol l}^{-1}$) and Pitressin (4 pressor units, containing approximately $0.6 \text{ nmol } \alpha\text{-MSH}$, $1.2 \times 10^{-7} \text{ mol l}^{-1}$). Qualitatively the results obtained with the two stimulants are similar in that corticosterone, 18-OH-B and aldosterone are all stimulated ($P < 0.05$ or better) whereas 18-OH-DOC is not; effects on DOC are variable. These results parallel those obtained with ACTH and posterior pituitary extract 1 (see Fig. 1). Quantitative differences between the two stimulants are attributable to the inclusion in Pitressin of further peptides (B and C, see Fig. 2) which also stimulate the rat zona glomerulosa cell. Values are means $\pm$ s.e.m., $n = 6$ throughout, except for Pitressin-stimulated DOC, $n = 2$.

peak A. Furthermore, examination of peak A on HVPE gives, on staining with Cd-ninhydrin²⁷, a major spot with identical mobility to α -MSH, in addition to more weakly staining neutral and acidic peptides. Overall, the structural and chromatographic data strongly indicate that the biological activity is derived from α -MSH.

Authentic α -MSH was therefore tested in both types of incubation system (Figs 4 and 5). Clearly the features of the response to α -MSH show many points in common with the response to Pitressin. In the first place, only glomerulosa cells are stimulated, and there is no effect on the fasciculata/reticularis cells (shown to respond normally to ACTH). Second, the pattern of the intact capsule steroid profile and its response to stimulation is similar to Pitressin (and to ACTH, see Fig. 1) in that corticosterone, 18-hydroxycorticosterone and aldosterone were stimulated, whereas DOC and 18-OH-DOC were not affected. Half maximal stimulation with synthetic α -MSH is obtained at about $10^{-8} \text{ mol l}^{-1}$ and with Pitressin at 100 mU per flask (Table I) which from its calculated α -MSH content would

yield about $3 \times 10^{-9} \text{ mol l}^{-1}$ of α -MSH. It seems likely from this data that the stimulatory potential of Pitressin is partly, perhaps 30%, due to α -MSH. In the case of posterior pituitary extract 1 it is also likely that the greater sensitivity of glomerulosa cells (compared with fasciculata/reticularis) to its stimulation may also be due to α -MSH in part, since the N-terminal ACTH assay, with which there is a 100% cross-reaction with α -MSH¹⁹ gave higher values than the C-terminal assay. However, it is also certain that this extract contained intact ACTH and possibly other factors to account for its greater potency when compared with Pitressin on the basis of its N-terminal reactivity (Table 1).

Earlier studies have suggested the stimulation of aldosterone production by α -MSH^{28,29}, and recently it has been shown that cortisol production by fetal human and sheep adrenal cells responds to α -MSH stimulation but this capacity is lost in the adult sheep^{30,31} (although conflicting results have been obtained with rhesus monkey fetal adrenal cells³²). It seems likely therefore that although the major site of steroidogenesis in the adult adrenal, the fasciculata cell, is not responsive to α -MSH, other cell types may retain this capacity.

Whether α -MSH functions as a trophic hormone on the zona glomerulosa in the adult is a matter for further investigation. Reported circulating levels of α -MSH in normal rats are of the order of $10^{-10} \text{ mol l}^{-1}$, below the threshold of stimulation in our experiments (see Fig. 4)³³. However, plasma levels will certainly vary, depending on the physiological condition of the animal, and in this connection it is particularly interesting to note that changes in sodium status in rats exert profound effects on pars intermedia histology and α -MSH content, and thus presumably on α -MSH secretion³⁴⁻³⁶. While it might be expected that a true physiological messenger should (in contrast to the effect of α -MSH) elicit a greater response on aldosterone than on corticosterone, experience with other stimulants such as potassium or angiotensin II added acutely has shown that such 'late pathway' effects are only revealed with the adoption of a harsher methodology (see for example ref. 37). It is also possible that there may be species variation in the response of the zona glomerulosa to MSHs in view of the relative lack of response of the human gland to α -MSH³⁸ and of the sheep gland to β -MSH³⁹.

The identities of the remaining UV peaks (B and C) which are coincident with biological activity are now being determined using the combination of purification and mass spectrometric methods described above.

We thank Drs E. C. Nice and M. J. O'Hare (Ludwig Institute for Cancer Research, Royal Marsden Hospital) for initial fractionation of Pitressin in their system²³. We also thank Dr Myron Miller, Veteran's Administration Hospital, Syracuse, New York) for the gift of posterior pituitary extract 1, Professor Lesley Rees for the radioimmunoassays on this material, and Dr D. M. Burley (Ciba-Geigy) for the gift of ACTH₁₋₂₄ (Synacthen). We are also grateful to MRC for grant support to G.P.V., and the MRC and SRC for grant support to H.R.M.

Received 2 November 1979; accepted 14 February 1980.

- Hanng, R., Tait, J. F. & Tait, S. A. S. *Endocrinology* **87**, 1147-1167 (1970).
- Müller, J. *Regulation of Aldosterone Biosynthesis* (Springer, Berlin, 1971).
- Blair-West, J. R. et al. *J. clin. Endocr.* **32**, 575-578 (1971).
- Vinson, G. P., Whitehouse, B. J., Goddard, C. & Sibley, C. P. *J. Endocr.* **81**, 5P-24P (1979).
- Spät, A. & Jozan, S. *J. Endocr.* **65**, 55-63 (1975).
- Palmore, W. P., Anderson, R. & Mulrow, P. J. *Endocrinology* **86**, 728-734 (1970).
- Urchhart, J. in *Handbook of Physiology* Vol. 4, Sect. 7 (eds Greep, R. O. & Astwood, E. B.) 133-157 (American Physiological Society, Washington, 1974).
- Ganong, W. F., Pemberton, P. L. & Van Brunt, E. E. *Endocrinology* **81**, 1147-1150 (1967).
- Palmore, W. P. & Mulrow, P. J. *Science* **158**, 1482-1484 (1967).
- Lee, T. C., Van der Wal, B. & de Wied, D. *J. Endocr.* **42**, 265-275 (1968).
- Solyom, J., Ludwig, E. & Vajda, A. *Acta physiol. acad. sci. hung.* **39**, 343-349 (1971).
- Williams, G. H., Rose, L. I., Dluhy, R. G., Dingham, J. F. & Lawler, D. J. *clin. Endocr.* **32**, 27-35 (1971).
- Palkovits, M., de Jong, W. & de Wied, D. *Neuroendocrinology* **14**, 297-309 (1974).
- Giroud, C. J. P., Stachenko, J. & Piletta, P. in *An International Symposium on Aldosterone* (eds Müller, A. F. & O'Connor, C. M.) 56 (Churchill, London, 1958).
- Venning, E. H., Lucis, O. J., Dyrenfurth, I. & Beck, J. C. in *The Human Adrenal Cortex* (eds Currie, A. R., Symington, T. & Grant, J. K.) 185-195 (Livingstone, Edinburgh, 1962).
- Kaplan, N. O. & Lipman, F. J. *biol. Chem.* **174**, 37-44 (1948).
- Douglas, J., Aguilera, G., Kondo, T. & Catt, K. J. *Endocrinology* **102**, 685-696 (1978).
- Richardson, M. C. & Schulster, D. J. *Endocr.* **55**, 127-139 (1972).
- Silman, R. E. et al. *J. Endocr.* **81**, 19-34 (1979).

- Vinson, G. P., Whitehouse, B. J., Goddard, C. & Sibley, C. P. *J. Steroid Biochem.* **11**, 175-183 (1979).
- Whitehouse, B. J. & Vinson, G. P. *J. Endocr.* **77**, 8P-9P (1978).
- Morris, H. R., Etienne, A. T., Dell, A. & Albuquerque, R. J. *Neurochem.* **34**, 574-582 (1980).
- O'Hare, M. J. & Nice, E. C. *J. Chromatogr.* **171**, 209-226 (1979).
- Morris, H. R. *FEBS Lett.* **22**, 257-260 (1972).
- Stone, J. V., Mordue, W., Batley, K. E. & Morris, H. R. *Nature* **263**, 207-211 (1976).
- Morris, H. R. & Dell, A. in *Instrumentation in Amino Acid Sequence Analysis* (ed. Perham, R. N.) (Academic, London, 1975).
- Heilmann, J., Barrolier, J. & Watzke, E. *Hoppe-Seyler's Z. physiol. Chem.* **309**, 219-220 (1957).
- Van der Wal, B. & de Wied, D. *Acta endocr.* **59**, 186-192 (1968).
- Page, R. B., Boyd, J. E. & Mulrow, P. J. *Endocr. Res. Commun.* **1**, 53-62 (1974).
- Glickman, J. A., Carson, G. D. & Challis, J. R. G. *Endocrinology* **104**, 34-39 (1979).
- Llanos, A. J., Ramachandran, J., Creasy, R. K., Rudolph, A. M. & Serón-Ferré, M. *Endocrinology* **105**, 613-617 (1979).
- Walsh, S. W., Norman, R. L. & Novy, M. J. *Endocrinology* **104**, 1805-1813 (1979).
- Thody, A. J., Penny, R. J., Clark, D. & Taylor, C. J. *Endocr.* **67**, 385-395 (1975).
- Duchen, L. W. J. *Endocr.* **25**, 161-168 (1962).
- Howe, A. & Thody, A. J. *J. Endocr.* **46**, 207-208 (1970).
- Kobayashi, Y. & Takema, M. *Cell Tissue Res.* **168**, 153-159 (1976).
- Aguilera, G. & Catt, K. J. *Endocrinology* **104**, 1046-1052 (1979).
- Birkhäuser, M., Gaillard, R., Riondel, A. M. & Zahnd, G. R. *Acta endocr.* **79**, 16-24 (1975).
- Blair-West, J. R. et al. *J. clin. Invest.* **41**, 1606-1627 (1962).

A newly identified population of presumptive microneurons in the cat retinal ganglion cell layer

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A large population of microneurons has recently been discovered in the rabbit retinal ganglion cell layer^{1,2}. These 'coronate' cells may represent a class of displaced amacrine cells^{1,2}. Like conventional amacrine cells³ they are swiftly and selectively destroyed⁴ by low concentrations of kainic acid, a neurotoxin⁵. No similar cell has been described in the cat retina, which is reported to contain 217–260,000 neurones of classical appearance⁶. These outnumber the 128–180,000 optic nerve fibres^{7,8} but it has been suggested that the excess comprise Nissl-staining glial cells⁹. We report here that, using the neurotoxic effects of kainic acid to test and confirm the neuronal nature of the classic neurone excess, a large additional population of at least 730,000 presumptive microneurons was revealed. They resemble rabbit coronate cells, do not project into the optic nerve and have been previously identified as presumed glia^{6,10,11}. Various lines of evidence for the neuronal nature of these cells is presented below, but synapses have not been demonstrated; subsequent reference to microneurons must therefore be regarded as presumptive.

The vitreous humor of cats anaesthetised with Nembutal was injected with either 12 or 120 nM of kainic acid in 10 μ l of saline. After 3 h the retina was removed, mounted and stained⁶.

As shown in Fig. 1, near the area centralis, α , β and larger γ ganglion cells¹² seemed to be normal at the lower dosage but, apart from one microglial cell, none of the remaining non-vascular profiles resembled those of normal retina, and all showed kainic-induced necrocytosis; the nuclei of many were pyknotic⁵. Small γ -mode neurones seemed scarce but the number of necrotic profiles was far in excess of their possible

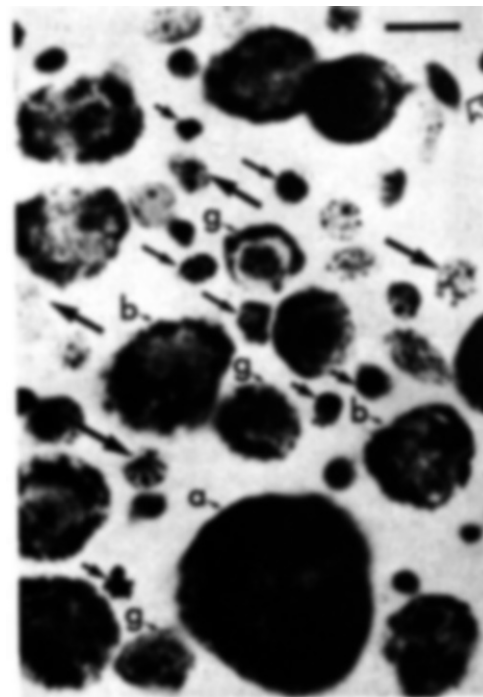


Fig. 1 Cells in the ganglion cell layer of the cat retina after an intraocular injection of 10 nM kainic acid. They are situated near the 2,500 classic neurones mm^{-2} isodensity line some 1.5 mm temporal of the area centralis. α (a), β (b) and the larger γ (g) Nissl-stained somas are apparent in relatively unchanged form. One γ cell near the centre of the figure has begun to degenerate and shows the characteristic nucleus with clumped chromophilic material. The large, medium and small arrows indicate profiles in progressively more advanced stages of degeneration. The final product is the pyknotic profile designated by the smallest unlabelled arrows. The open arrow indicates a classic glial cell. The degenerating profiles of this region must represent small cells because the densities of large and medium-sized cells are normal. The number of kainic-sensitive cells far exceeds the possible number of degenerate γ cells. Alcohol/formalin-fixed material. Scale bar, 10 μm .

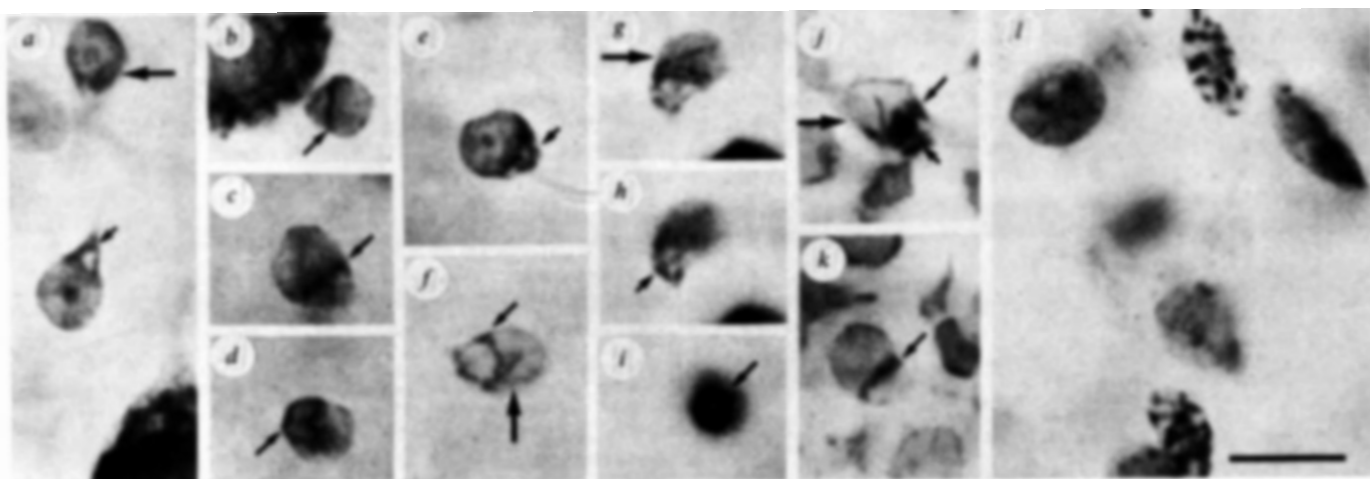


Fig. 2 *a-i*, Cells from the untreated cat ganglion cell layer resembling those which are destroyed by kainic acid and have previously been presumed to be glia. Small arrows: RNA-specific basophilia in free cytoplasmic granules; medium arrows: similar material at the juxtannuclear region of the cell's cytoplasmic pole; large arrows: basophilic nuclear invaginations. The nucleolus is obvious in cells of *a* and *e*. The subnuclear ring of basophilia is apparent in *d*, *f* and *i*; in *i* it was below the nucleus. *j*, *k*, Cells of the amacrine layer with features resembling those of kainic-sensitive profiles in the ganglion cell layer (*a-i*). *l*, Two types of glial nucleus from the nerve fibre layer; one shows clumped, the other dispersed, chromatin. These are from an eye injected with 120 nM of kainic acid and enucleated after 3 h, but their appearance is within the normal range. Cells such as those in *a-k* were destroyed by this treatment although they lie deeper within the retina. Alcohol/formalin fixed. Scale bar 10 μm .

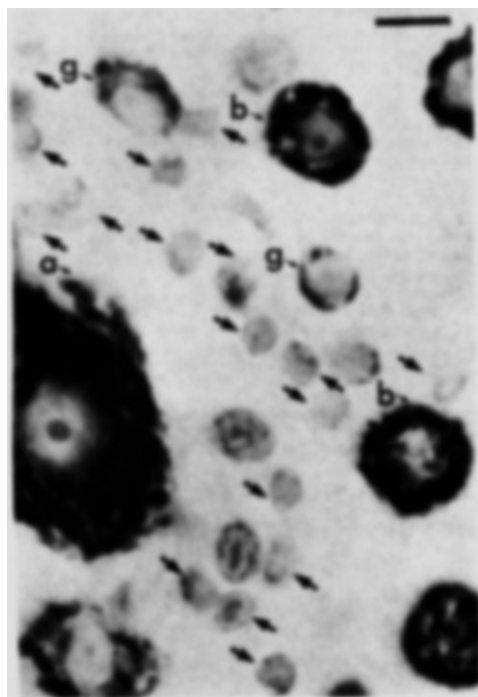


Fig. 3 A lightly Nissl-stained region of normal retina somewhat more peripheral than that of Fig. 1. The somas of Nissl-stained α (a), β (b) and γ (g) mode neurones are apparent. The small arrows indicate the profiles of cells previously identified as presumed glia^{7,12,13}. A large number of these cells are present, as would be expected in a region of normal retina similar to that shown in Fig. 1. However, no such profiles with relatively clear nuclei and juxtanuclear basophilia can be seen in Fig. 1. A quantitative analysis (see text) shows the majority of the kainic-sensitive profiles to correspond to the small, pale profiles shown here. These presumed glia^{7,13} are thus sensitive to the influence of a neurotoxic compound not known to have gliotoxic effects. Alcohol/formalin-fixed material. Scale bar 10 μm .

contribution; the surprisingly large population of pyknotic cells was particularly obvious in the periphery, where ganglion cells are less common.

A 0.013-mm² area near that shown in Fig. 1 contained 75 non-vascular profiles. Of these, 2 were classic glia with obvious nuclear chromatin, 2 were α -mode cells, 15 β -mode cells and 8 γ -mode cells; the remainder were unlike any profiles of normal retina. Because this region in untreated retina has a γ -cell population equal to the combined α - and β -mode populations¹³ it is estimated that nine of the novel profiles represent degenerate classic neurones of the γ mode. Thus, 34 profiles (45%) represent classic neurones; 2 (3%) were classic glia and the remaining 39 (52%) represent cells not previously included in the neurone count but which have been destroyed by a neurotoxic compound.

In normal retina this area (Fig. 5A of ref. 6) contains a similar proportion (43%) of classic neurones, but the remaining 57% of profiles are presumed to be glia. The most common of these 'presumed glia' lack the conspicuous nuclear chromatin of classic glia and often possess a prominent nucleolus⁶. In kainic-treated retina only 3% of profiles in the ganglion cell layer were recognisable as glia; the remaining 54% of presumed glia were missing. There was instead a corresponding proportion of 52% residual kainic-damaged profiles which we conclude represents the necrotic presumed glia of the normal cat retina; their absolute densities are similar at 2,800 mm⁻² and 2,960 mm⁻² (ref. 6).

Kainic acid does not seem to be gliotoxic¹⁴; early glial response to kainic acid^{15,16} resembles that to glutamate^{3,17,18} and is reported to involve swelling but not necrosis and pyknosis. The exposed glial cells of the fibre layer and perivascular glia were unchanged in appearance even at the 10 times higher dose of kainic acid (Fig. 2I), as were glia in the ganglion cell and inner plexiform layers. Müller cell bodies and feet seemed normal, without the damage we observe with DL-amino adipic acid, a known gliotoxic compound¹⁹. On this basis, we conclude that the majority of the presumed glia in the normal cat ganglion cell layer are, in fact, neurones.

Figure 3 shows a lightly stained region of normal cat retina; the majority of profiles correspond to the presumed glia of previous reports^{6,9,11}. The pear shape of some cells (Fig. 2), the juxtanuclear basophilia, basophil nuclear invaginations and

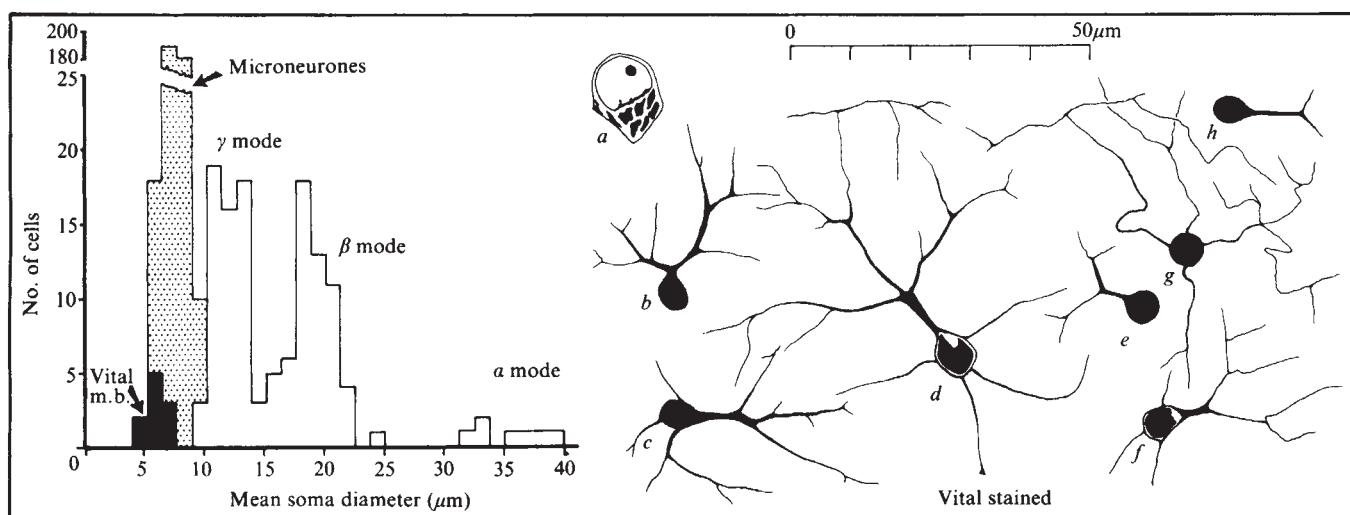


Fig. 4 The soma diameter spectrum illustrates the size range of non-vital stained cells in the various classic neurone modes of peripheral cat retina subject to supravital methylene blue staining and non-vital counterstaining. In addition, the stippled regions represent the presumed microneurone spectrum previously identified as presumed glia. The location in the soma size spectrum of some supravital methylene blue-stained microneurones is indicated in black. The partially stained unequivocal neurones *b* to *h* occupy the lower range of the microneurones. A medium-sized γ soma is shown at *a* for comparison. Neurones occupying a region of the diameter spectrum significantly outside the γ mode of classic neurones have not previously been reported. Their presence shows that cells in the class labelled 'microneurones' are not too small to be neurones of the cat retinal ganglion cell layer.

cytoplasmic granules, size range and restricted cytoplasm are similar to those of rabbit coronate cells². Some possess the 'crown' of juxtannuclear basophilia which led to the term 'coronate' in rabbit (Fig. 2*d, f, i*); its basophilia to Gallocyanin-lake is confirmed to be RNA-specific in the cat by its elimination with prior ribonuclease incubation, a result similar to that seen in the rabbit². The complex nuclear form^{20,21}, its basophilic invaginations²¹ and cytoplasmic RNA basophilia²² are characteristically neuronal^{20,21}.

These cells are smaller than any neurones previously described in the cat retinal ganglion cell layer and are different from reported cat displaced amacrine cells²³. Supravital methylene blue-stained retinas were examined for examples of their dendritic trees. Figure 4 shows the diameter range of soma profiles for neurones in such preparations. Dendritic trees are shown only for cells with very small, 5–7 µm, somata well displaced from the γ-mode population. None are completely stained but the variety of form suggests considerable heterogeneity; their vital staining offers additional evidence for their neuronal nature²⁴. Many possess one main dendrite which branches close to the soma. No axons or axon-hillocks were observed and the cells do not fill with horseradish peroxidase from the optic nerve¹³.

In peripheral retina the presumptive microneurones form 75% of the profiles in the retinal ganglion cell layer but their density is lower, 1,550 mm⁻². In a 470-mm² cat retina⁶ this suggests a population of at least 730,000 microneurones. Adding the 217,000 classic neurones⁶ we estimate a population of up to 10⁶ neurones in the cat ganglion cell layer. This is so far in excess of even the largest optic nerve fibre counts that it precludes the possibility of a significant proportion of the microneurones being ganglion cells. That such a large population has remained undiscovered for so long suggests that they are, like the interplexiform cell²⁵, only rarely impregnated by the capricious Golgi stain.

Two classes of neurone which do not project into the optic nerve are recognised in the retinal ganglion cell layer—cells of Marengi^{26,27} and displaced amacrine cells²⁸. Cells of Marengi, unlike the microneurones of cat and rabbit retina, possess an axon. However, cells similar to microneurones do occur in the amacrine layer (Fig. 2*j, k*) of both species. Rabbit coronate cells², cat microneurones and non-displaced amacrine cells³ are all sensitive to kainic acid. It is concluded that the microneurones represent a class of 'displaced' amacrine cell. There are additional reasons for assuming this in the rabbit².

Displaced amacrine cells are regarded as common in non-mammalian species²⁸ but have only occasionally been identified in mammals^{23,28–30}. Their large number in the bird retina^{31,32} has been well demonstrated³³. Now cat, rabbit^{1,2} and rat³⁴ are also known to possess a large population of neurones in the ganglion cell layer which are not ganglion cells. No distinctive role has been postulated for either displaced amacrine cells or the microneurone population. The relative uniformity of microneurone density across the retina suggests that these cells function in relation to a fixed-size module of inner nuclear layer neurones.

Received 7 October 1979; accepted 5 February 1980.

1. Vaney, D. I. *J. comp. Neurol.* (in the press).
2. Hughes, A. & Vaney, D. I. *J. comp. Neurol.* **189**, 169–190 (1980).
3. Coyle, J. T., Biziere, K. & Schwarcz, R. in *Kainic Acid as a Tool in Neurobiology* (ed. McGeer, E. G.) 177–188 (Raven, New York, 1978).
4. Hughes, A. & Wieniawa-Narkiewicz, E. (in preparation).
5. Olney, J. W. in *Kainic Acid as a Tool in Neurobiology* (ed. McGeer, E. G.) 95–122 (Raven, New York, 1978).
6. Hughes, A. *J. comp. Neurol.* **163**, 107–128 (1975).
7. Stone, J. & Campion, J. E. *J. comp. Neurol.* **180**, 799–806 (1978).
8. Hughes, A. & Wässle, H. *J. comp. Neurol.* **169**, 171–184 (1976).
9. Stone, J. *J. comp. Neurol.* **180**, 753–772 (1978).
10. Stone, J. *J. comp. Neurol.* **124**, 337–352 (1965).
11. Wässle, H., Levick, W. R. & Cleland, B. G. *J. comp. Neurol.* **159**, 419–438 (1975).
12. Boycott, B. B. & Wässle, H. *J. Physiol., Lond.* **240**, 397–419 (1974).
13. Hughes, A. *J. comp. Neurol.* (submitted).
14. Coyle, J. T. *Trends Neurol. Sci.* **1**, 132–135 (1978).
15. Coyle, J. T., Molliver, M. E. & Kuhar, M. J. *J. comp. Neurol.* **185**, 302–323 (1979).
16. Olney, J. W., Fuller, T. & De Gubareff, T. *Brain Res.* **176**, 91–100 (1979).
17. Olney, J. W. *J. Neuropath. exp. Neurol.* **28**, 455–474 (1971).
18. Van Harreveld, A. & Fitkova, E. *Expl. molec. Path.* **15**, 61–81 (1971).

19. Olney, J. W., Ho, O. L. & Rhee, V. *Expl. Brain Res.* **14**, 61–76 (1971).
20. Smallwood, W. M. & Rogers, G. G. *J. comp. Neurol.* **18**, 45–56 (1968).
21. Peters, A., Palay, S. L. & Webster, H. de F. in *The Fine Structure of the Nervous System* (Saunders, Philadelphia, 1976).
22. Koenig, H. in *Morphological and Biochemical Correlates of Neural Activity* (eds Cohen, M. M. & Snider, R. S.) 39–56 (Harper and Row, New York, 1961).
23. Gallego, A. *Vision Res. Suppl.* **3**, 33–50 (1971).
24. Harris, J. E. & Peters A. Q. *J. microsc. Sci.* **94**, 113–124 (1953).
25. Boycott, B. B., Dowling, J. E., Fisher, S. K., Kolb, H. & Laties, A. M. *Proc. R. Soc. B191*, 353–368 (1975).
26. Gallego, A. & Cruz, J. *Science* **150**, 1313–1314 (1965).
27. Dawson, W. W. & Lieberman, H. *Proc. ARVO spring meet.*, Sarasota, 117 (1978).
28. Cajal, S. Ramon Y. in *Histologie du système Nerveux* Vol. II (Consejo Superior de Investigaciones Científicas, Madrid, 1955).
29. West, R. W. *J. comp. Neurol.* **168**, 355–378 (1976).
30. Perry, V. H. *Proc. R. Soc. B204*, 363–375 (1979).
31. Bingelli, R. L. & Paule, W. J. *J. comp. Neurol.* **137**, 1–18 (1969).
32. Nishimura, Y., Inoue, Y. & Shimai, K. *Expl. Neurol.* **64**, 44–60 (1979).
33. Galvez, J. E. G., Puellas, L. & Pradu, C. *Expl. Neurol.* **56**, 151–157 (1977).
34. Hughes, A. *J. comp. Neurol.* **176**, 263–268 (1977).

A role for collagen in the pathogenesis of muscular dystrophy?

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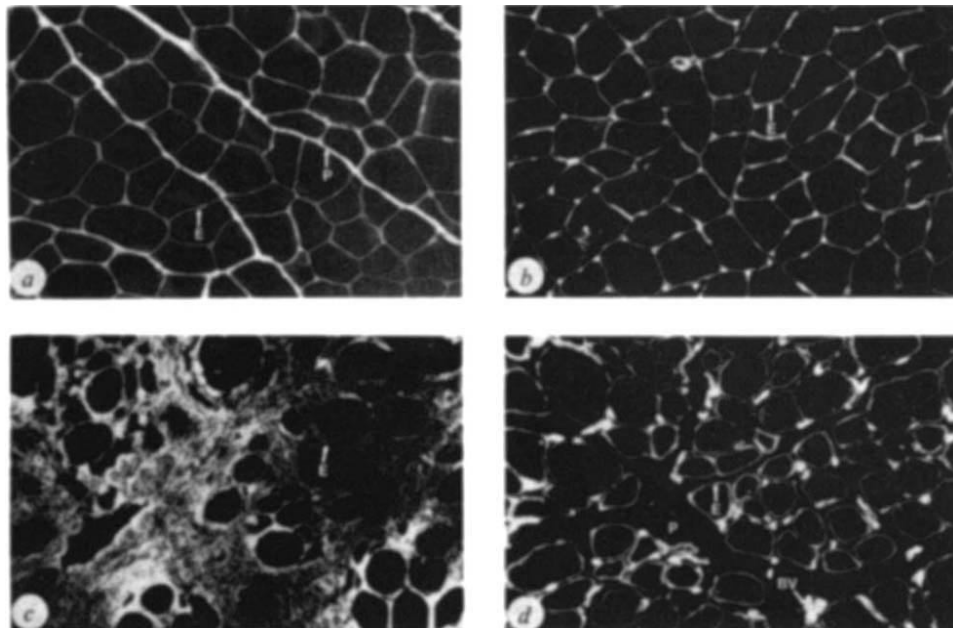
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The extensive proliferation of connective tissue in muscular dystrophy caused Duchenne¹ to term it 'paralyse myosclerosique'. Surprisingly, there has been little interest in the pathogenesis of this marked fibrosis or of the fat replacement in dystrophic muscle. The fibrosis has generally been considered secondary, with various hypotheses² to explain the fundamental cause of muscular dystrophy. Several authors have commented on the increased endomysial stroma early in the disease before any apparent muscle degeneration^{3,4} and have suggested that there might be an aberration in the production of connective tissue in muscular dystrophy and that the thickening pericellular reticulum would adversely affect muscle nutrition^{5,6}. Ionasescu and his collaborators found an over-production of connective tissue with a concomitant decrease in muscle protein synthesis both by polyribosomes from skeletal muscle and by tissue culture of muscle tissue and skin fibroblasts from patients suffering from Duchenne muscular dystrophy^{7–9}. Thomson *et al.*¹⁰ have observed in tissue culture that dissociated muscle from patients with Duchenne and Becker muscular dystrophy will form unusual clusters of 'sticky' cells, which they suggested may reflect an abnormal collagen production. These findings have cast doubts on the interpretation that the extensive connective tissue proliferation characteristic of Duchenne muscular dystrophy, and also seen in limb-girdle, Becker and congenital dystrophies, is simply due to a compensatory replacement of the wasting muscle¹¹ but infers a more primary role for the connective tissue collagen. Only recently has the role of connective tissue in developing muscle come to be appreciated^{12–14}. Moreover, immunofluorescent techniques^{14–16}, now allow investigation of the various types of collagen present in skeletal muscle. We report here on the localisation and change in proportion of collagen types I, III, IV and V in muscle from patients with various forms of neuromuscular disease, and propose a more positive role for collagen.

Needle biopsies were obtained from 15 patients (2–12 yr old) suffering from Duchenne and limb-girdle muscular dystrophy as assessed by clinical, and histological criteria¹¹. Collagen types I,

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Fig. 1 Immunofluorescence staining of transverse sections ($\times 60$) of normal (*a, b*) and dystrophic human muscle (*c, d*) with antibodies to type III (*a, c*) and type IV (*b, d*) collagen. Cryostat sections were stained by the indirect procedure using a fluorescein-labelled anti-rabbit or anti-goat IgG for the second stain. The excessive deposition of type III collagen in muscular dystrophy is readily observed when compared with normal muscle (*a, c*). This increase in type III collagen is distributed in the perimysium (P) and the endomysium (E). The apparent endomysial basement membrane thickening is seen when comparing normal muscle (*b*) with diseased muscle (*d*) stained with anti-type IV antibodies. Note also the more prominent blood vessels (BV) in the diseased muscle. Less dramatic changes are observed with sections stained with antibodies to types I and V (not shown).



III, and IV were localised by fluorescent antibody staining techniques¹⁵ on cryostat sections. The antibodies were raised in rabbits or goats using purified human collagens¹⁷. Antisera were rendered type specific by passage through a series of immunoabsorbent columns with types I–V collagens covalently coupled to Sepharose-4B. The antibodies were assayed by passive haemagglutination for activity and specificity. No cross-reactivity was observed at the lowest dilution used (1/4) with antibody titres ranging over 1/64–1/1,024. The anti-collagen antibodies used for the immunofluorescence staining were diluted to give titres of 1/50–1/100; the fluorescein-conjugated anti-rabbit IgG (Wellcome) and fluorescein-conjugated anti-goat IgG (Miles) were used as recommended by the suppliers.

The distribution of these various collagen types in normal human muscle paralleled that previously found in bovine and chick skeletal muscle^{14–16}. Antibodies to type I collagen stained the epimysium and tendon bundles with slight staining of the perimysium; anti-type III, although staining the epimysium, exhibited a much greater affinity for the perimysium and endomysium between the muscle fibres (Fig. 1*a*); anti-type IV stained the contours of individual muscle fibres intensely, as would be expected for this basement membrane collagen (Fig. 1*b*); anti-type V was also present in the endomysial basement membrane surrounding each muscle fibre, although there was also slight fluorescence in the perimysium.

The most striking feature of Duchenne muscle was the vast anti-type III staining in the peri- and endomysial tissue around the muscle fibres, some of which were small and either atrophying or regenerating and others which were hypertrophied (Fig. 1*c*). The enhanced type III staining was present even in pre-

clinical cases and was a consistent feature of all patients examined. In later stages of the disease the relative increase in type III collagen was proportionately less due to the massive adipose tissue replacement. The vast proliferation of type III collagen in muscle is typical of the response of tissue to injury, as observed in wound healing¹⁸.

The prominent variation in fibre size characteristic of muscle from patients with Duchenne and limb-girdle muscular dystrophies could readily be seen when the endomysial basement membrane anti-type V collagen was stained with anti-type IV (Fig. 1*d*) and anti-type V collagen. Further, the staining of the basement membrane with anti-type IV and V collagen was occasionally more intense around smaller muscle fibres. Whether this reflects a thicker basement membrane or a more extensive infolding of the basement membrane coincident with myofibrillar loss¹⁹ remains to be resolved, perhaps with electron microscopy. Many of the circular profiles which stain prominently with anti-type IV collagen are small capillaries (see Fig. 1*d*). They are much more marked in biopsied muscle in cases of Duchenne muscular dystrophy, which agrees with Jerusalem's observation of reduplicated basement membrane forming two or more continuous or discontinuous layers around the vessel and of an increased mean capillary area²⁰ in Duchenne dystrophy. On the other hand, some of the small fluorescent profiles may be surviving remnants of muscle basement membrane^{21,22}.

The presence of thickened basement membrane and the observation of splits within a few of the muscle fibre bundles from Duchenne and limb-girdle patients are characteristic of muscle disease and muscle injury^{21,23–25}. The appearance of splits in hypertrophied and whorled fibres may denote an early stage in the breakdown of the muscle fibre. Our present finding of type III and IV collagen in these splits (Fig. 2) suggests that collagen may play a part in this process. The recognition of the importance of connective tissue components in muscle disease should provide further impetus in the study of the aetiology of muscular dystrophies.

This investigation was supported in part by the Arthritis and Rheumatism Council (VCD), Muscular Dystrophy Association of Canada, the Muscular Dystrophy Group of Great Britain (HRS) and the Medical Research Council (MD). We thank Mrs C. Hutson for technical assistance.

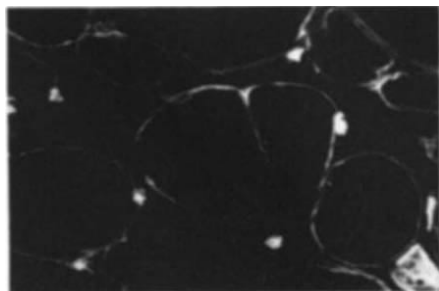


Fig. 2 Transverse section of dystrophic muscle ($\times 146$) stained as in Fig. 1 with anti-type IV collagen. Note the presence of staining in the split within the muscle fibre.

Received 28 November 1979; accepted 18 February 1980.

1. Duchenne, G. B. *Archs gén. Méd.* 6, 5 (1868).
2. Rowland, L. P. *Arch. Neurol.* 33, 315–321 (1976).
3. Pearson, C. M. in *Muscular Dystrophy in Man and Animals* (eds Bourne, G. H. & Golarz, M. N.) (Hafner, New York, 1963).

4. Bell, C. & Conen, P. *J. neurol. Sci.* **7**, 529–544 (1968).
5. Bourne, G. H. & Golarz, M. N. *Nature* **183**, 1741–1743 (1959).
6. Cazzato, G. *Eur. Neurol.* **1**, 158–179 (1968).
7. Ionasescu, V. H., Zellweger, H. & Conway, V. *Archs Biochem. Biophys.* **144**, 51–58 (1971).
8. Ionasescu, V. H., Zellweger, H., Ionasescu, V. I., Braud, C. L. & Cancilla, P. A. *Acta neurol. scand.* **54**, 241–247 (1976).
9. Ionasescu, V., Braud, C. L., Zellweger, H., Ionasescu, R. & Burmeister, L. *Acta neurol. scand.* **55**, 407–417 (1977).
10. Thomson, E. J., Yasin, R., Van Beers, G., Nurse, K. & AlAni, S. *Nature* **268**, 241–243 (1977).
11. Dubowitz, V. *Muscle Disorders in Childhood* (Saunders, London, 1978).
12. Lipton, B. H. *Dev. Biol.* **61**, 153–165 (1977).
13. Wolpert, L. *Scient. Am.* **239**, 124–137 (1978).
14. Bailey, A. J., Shellswell, G. B. & Duance, V. C. *Nature* **278**, 67–69 (1979).
15. Duance, V. C., Restall, D. J., Beard, H., Bourne, F. J. & Bailey, A. J. *FEBS Lett.* **79**, 248–252 (1977).
16. Duance, V. C., Black, C. M., Dubowitz, V., Hughes, G. & Bailey, A. J. *Muscle Nerve* (in the press).
17. Bailey, A. J., Duance, V. C., Sims, T. J. & Beard, H. K. *Front. Matrix Biol.* **7**, 49–59 (1979).
18. Bailey, A. J., Bazin, S., Sims, T. J., Le Lous, M., Nicoletis, C. & Delaunay, A. *Biochim. biophys. Acta* **405**, 412–421 (1975).
19. Fitchett, D. H., Scott, J., Stephens, H. R. & Peters, T. J. *Cardiovasc. Res.* **13**, 260–268 (1979).
20. Jerusalem, F., Engel, A. G. & Gomez, M. R. *Brain* **97**, 115–122 (1974).
21. Mair, W. G. & Tome, F. M. *Atlas of the Ultrastructure of Diseased Human Muscle*, 140 (Churchill, London 1972).
22. Carpenter, S. & Kapati, C. *Brain* **102**, 147–161 (1979).
23. Neville, H. in *Muscle Biopsy: A Modern Approach* (eds Dubowitz, V. & Brooke, M. H.) Ch. 12 (Saunders, London, 1973).
24. Cullen, M. J., Appleyard, S. T. & Bindoff, L. *Ann. N.Y. Acad. Sci.* **317**, 440–465 (1978).
25. Vracko, R. *Biology and Chemistry of Basement Membranes* (ed. Kefalides, N. A.) 165–176 (Academic, New York, 1978).

Haloperidol-induced catalepsy is mediated by postsynaptic dopamine receptors

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Antipsychotic or neuroleptic drugs, which block central dopamine receptors, produce a behavioural state in animals in which they fail to correct externally imposed postures. This is referred to as catalepsy. Previous lesion studies have shown that the dopamine receptors in the striatum are involved in this neuroleptic-induced catalepsy^{1–5}. Dopamine receptors, identified by the specific, high-affinity binding of the potent neuroleptic haloperidol, have been shown to be equally distributed postsynaptically on striatal neurones and presynaptically on cortico-striatal terminals^{6–9}. Because the electrolytic lesioning studies^{1–5} have unavoidably damaged both pre- and postsynaptic striatal dopamine receptors, it is not known whether these two receptors are separately involved in neuroleptic-induced catalepsy. Using kainic acid and cortical ablation to destroy postsynaptic and presynaptic dopamine receptors, respectively^{6–9}, the present study demonstrates that the cataleptic effects of haloperidol are apparently mediated by dopamine receptors localised postsynaptically on striatal neurones.

Ten male Wistar hooded rats weighing about 350 g received 6 nmol of kainic acid injected stereotactically into each striatum^{10,11}. Ten control animals received vehicle injections only. In another eight rats the parietal and frontal cortex was removed. Nine control animals received sham operations. All the rats received a prophylactic subcutaneous injection of aquacaine (0.2 ml) and an intraperitoneal (i.p.) injection of diazepam (5 mg per kg) immediately following surgery (diazepam reduces the possibility of extrastriatal damage in rats injected with kainic acid^{12,13}). All animals were allowed to recover for 4 weeks before testing for catalepsy. After testing, the animals were perfused intracardially with 10% formol saline and their brains examined histologically. Consistent with previous reports^{10,11,14,15}, the kainate-lesioned rats showed a considerable loss of neuronal cell bodies in the dorsal two-thirds of the striatum, whereas very little extrastriatal damage was apparent.

In all rats with cortical ablation there was no destruction below the level of the corpus callosum.

The cataleptic effect of the central dopamine receptor blocker haloperidol (1 mg per kg) on kainate-lesioned and cortex-ablated rats is shown in Fig. 1. Catalepsy was measured by gently placing the animal's forepaws on a horizontal bar located 8.0 cm above the floor and timing the period before the animal placed at least one paw on the floor. If an animal remained on the bar for more than 5 min, it was removed and a score of 300 s was given. After saline injections, rats in all groups typically removed their paws from the bar within 20 s. The decorticate rats, however, showed slightly higher descent latencies under saline compared with their control group ($P < 0.05$). This may be due to an increased emotionality, as they defaecated substantially more than controls during testing. Injection of haloperidol induced considerable catalepsy in the control animals (Fig. 1). Haloperidol-induced catalepsy was almost totally blocked in the kainate-lesioned rats ($P < 0.01$). By contrast, haloperidol tended to increase catalepsy in decorticate rats, although this increase did not reach significance ($P < 0.07$).

It has previously been demonstrated^{6–9} that kainic acid-induced striatal lesions destroy about 40% of ³H-haloperidol binding sites in the striatum, demonstrating postsynaptic localisation on intrinsic striatal neurones. Decortication, which removes the cortico-striatal tract, resulted in a similar reduction of ³H-haloperidol binding, showing presynaptic localisation of dopamine receptors on cortico-striatal afferents. Although previous studies^{1–5} have shown that haloperidol-induced

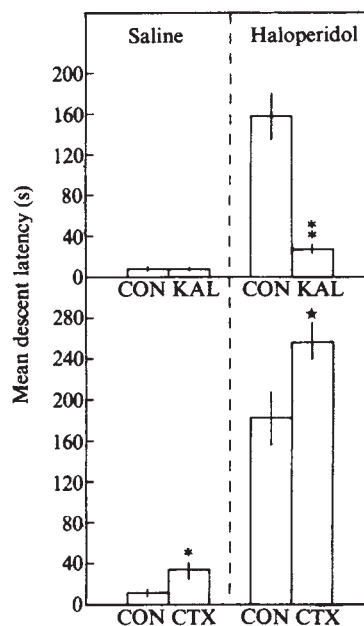


Fig. 1 Catalepsy induced in control (CON), kainate-lesioned striatal (KAL) and cortically ablated (CTX) rats. KAL rats ($n = 10$) were injected with 6 nmol kainate dissolved in 1.0 μ l of phosphate-buffered isotonic saline solution, pH 7.2, as described elsewhere^{10,11}. CON rats ($n = 10$) received infusions of the vehicle. CTX rats ($n = 8$) were anaesthetised with sodium pentobarbital (50 mg per kg i.p.) and positioned on a Kopf stereotaxic instrument. The skin was reflected, the calvaria overlying the parietal and frontal cortex was removed, and the underlying cortex was removed to the level of the corpus callosum by aspiration; bleeding was controlled by gelfoam. CON rats ($n = 9$) received sham operations. The data represent the pooled mean results of four catalepsy tests on each rat carried out at 1-h intervals following drug administration. Haloperidol was dissolved in 0.9% saline and injected i.p. at 1 mg per kg. All animals were injected with either haloperidol or saline in a randomly paired fashion. One week later, the other drug solution was administered. The experiment was run blind insofar as the drug solutions were coded. Control and lesioned groups differed at the following levels of significance using a two-tailed Mann-Whitney U test¹⁶: ★, 7%; *, 5%; **, 1%.

catalepsy seems to be mediated by dopamine receptors in the striatum, the electrolytic lesioning techniques used have been unable to examine specifically the roles of these two dopamine receptors in mediating the behavioural response. My results show that the cataleptic response to haloperidol injection is almost completely abolished in kainic acid-lesioned rats. The marginal increase in catalepsy induced by haloperidol in these animals may be due to the fact that destruction of the striatal interneurons is incomplete. If anything, haloperidol is more effective in inducing catalepsy in decorticate animals. This suggests that haloperidol-induced catalepsy requires the binding of haloperidol to postsynaptic dopamine receptors in the striatum⁶⁻⁹. The present findings may have relevance for understanding differences in antipsychotic potency of neuroleptic drugs in diseases with cortical and/or striatal atrophy.

I thank Professors R. F. Mark and I. Creese and Drs I. G. Morgan, D. Ehrlich, M. Pisa and I. J. Faulks for helpful comments, Roche (Australia) for the gift of diazepam, and Janssen (Belgium) for the gift of haloperidol. The author is an ANU Research Scholar.

Received 15 November 1979; accepted 5 February 1980.

- Arushanian, E. B. *Adv. Pharmac. Ther.* **5**, 107-116 (1978).
- Costall, B. & Olley, J. E. *Neuropharmacology* **10**, 297-306 (1971).
- Fog, R., Randrup, A. & Pakkenberg, H. *Psychopharmacology* **18**, 346-356 (1970).
- Honma, T. & Fukushima, H. *Jap. J. Pharmac.* **28**, 231-238 (1978).
- Koffer, K. B., Berney, S. & Hornykiewicz, O. *Eur. J. Pharmac.* **47**, 81-86 (1978).
- Creese, I., Usdin, T. & Snyder, S. H. *Nature* **278**, 577-578 (1979).
- Garau, L., Govoni, S., Stefanini, E., Trabucchi, M. & Spano, P. F. *Life Sci.* **23**, 1745-1750 (1978).
- Govani, S. *et al. Neurosci. Lett.* **8**, 207-210 (1978).
- Schwartz, R., Creese, I., Coyle, J. T. & Snyder, S. H. *Nature* **271**, 765-768 (1978).
- Mason, S. T., Sanberg, P. R. & Fibiger, H. C. *Science* **201**, 352-355 (1978).
- Sanberg, P. R., Pisa, M. & Fibiger, H. C. *Pharmac. Biochem. Behav.* **10**, 137-144 (1979).
- Ben-Ari, Y. *et al. Eur. J. Pharmac.* **52**, 419-420 (1978).
- Ben-Ari, Y., Tremblay, E., Ottersen, O. P. & Naquet, R. *Brain Res.* **165**, 362-365 (1979).
- Coyle, J. T. & Schwartz, R. *Nature* **263**, 244-246 (1976).
- McGeer, E. G. & McGeer, P. L. *Nature* **263**, 517-519 (1976).
- Siegel, S. *Nonparametric Statistics for Behavioral Sciences* (McGraw-Hill, New York, 1956).

Cell-free synthesis of enterotoxin of *E. coli* from a cloned gene

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In a variety of mammalian species including humans the newborn young are particularly susceptible to diarrhoeal disease brought about by toxin-producing strains of the common gut bacterium, *Escherichia coli*¹. Two types of enterotoxin have been described². The first, heat-labile toxin (LT), sharing partial antigenic identity with cholera toxin^{2,3} is comprised of two subunits having molecular weights of 25,500 and 11,500⁴ and, like cholera toxin, stimulates adenyl cyclase activity in the gut⁵. The second type, heat-stable toxin (ST), is a low-molecular weight protein which is excreted into the medium during the growth of ST-elaborating strains. Unlike LT, ST is poorly antigenic and for this reason the identification of ST-producing strains has relied on an assay involving the intragastric injection of infant mice, a test specific for ST⁶. Although many strains produce both ST and LT, strains producing ST alone are especially common in *E. coli* diarrhoeal disease of calves^{7,8}, pigs² and have also been implicated in outbreaks of diarrhoea in humans^{9,10}. Recent evidence suggests that ST activates guanyl cyclase in the gut^{11,12} and its molecular weight has been estimated to be 4,400-5,100 (ref. 13). We have now applied gene cloning techniques to the isolation of ST genes from two porcine *E. coli* isolates and report here the identification of the protein

product of molecular weight 7,000, synthesised in an *in vitro* system directed by the cloned DNA template. This *in vitro* gene product elicits a typical ST enterotoxin response in the infant mouse assay and is most probably synthesised as a 10,000 molecular weight precursor, as might be expected for an exported protein. The gene cloning data also provide evidence that the ST genes and their surrounding transposon structures are probably identical in both porcine and bovine strains of enteropathogenic *E. coli*.

The ability to produce either LT or ST, or both enterotoxins, has been shown in many instances to be a plasmid-mediated trait¹⁴⁻¹⁶. So, Heffron and McCarthy¹⁷ have used molecular cloning techniques to show that the plasmid-borne ST-coding region from a bovine *E. coli* isolate (B41) is located within a transposable DNA element (transposon Tn1681), the structural gene being flanked by inverted repeats of the IS1 insertion sequence.

We have found that commencing with two ST-elaborating porcine isolates (P288 and P310) of *E. coli*, the ST-coding region can be isolated on a *Pst*I endonuclease fragment of molecular weight (MW) 1.05×10^6 , cut symmetrically from within the IS1 sequences of the transposon¹⁸. Since the restriction endonuclease maps of the two porcine *Pst*I fragments are identical in number of sites and size of fragments with the data for the bovine ST gene (ref. 17; M. So, personal communication) we surmise that the ST genes and their transposon structures are identical in all three strains.

The ST-DNA contained within the *Pst*I fragment obtained from the porcine P310 isolate was further subcloned into plasmid pACYC184 (ref. 19). The central *Hae*III fragment was 'flush-end' ligated into plasmid pACYC184 which had been digested with *Eco*RI and the cohesive ends filled in by treatment with avian myoblastosis reverse transcriptase. This results in the regeneration of *Eco*RI sites at the termini of the original *Hae*III fragment. The recombinant plasmid pRIT 10211 so-formed was further manipulated to obtain pRIT 10220 by excising the ST-coding region with *Eco*RI and *Taq*I endonucleases and ligating the resulting fragment into the plasmid vector pBR322 (ref. 20) which had been digested with *Eco*RI and *Cl*aI. In this construction all IS1-sequence DNA has been removed from one side of the ST-DNA insert. The relevant structures of pRIT 10211 and pRIT 10220 are shown in Fig. 1.

These recombinant plasmids were then examined for their ability to stimulate protein synthesis in an *in vitro* transcription-translation system prepared from ST-free *E. coli*²¹. After incubation of plasmid DNA with a reconstituted extract of *E. coli* in the presence of ³H-leucine, newly-synthesised proteins coded by the plasmid were separated by polyacrylamide gel

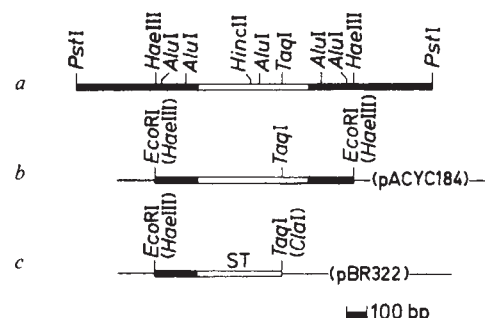


Fig. 1 Restriction endonuclease maps of recombinant ST plasmids. a, Map of the *Pst*I ST-DNA fragment isolated from strain P310 (compare ref. 17). The shaded bars indicate the extent of the IS1 sequence flanking the ST structural gene. b, pRIT 10211: the central *Hae*III ST-DNA fragment has been inserted at the *Eco*RI site of plasmid vector pACYC184. c, pRIT 10220: the *Eco*RI-*Taq*I ST-DNA fragment from pRIT 10211 subcloned into plasmid vector pBR322. The position of the ST gene is inferred from the fact that insertion of a foreign DNA sequence at the *Hinc*II site destroys ST gene activity and that both central *Alu*I fragments are necessary for ST biosynthesis¹⁷.

electrophoresis in the presence of SDS and subsequently revealed by autoradiography. Figure 2a shows that both pRIT 10211 and pRIT 10220 stimulate the synthesis of a protein of molecular weight 10,000 which is absent from the controls.

To identify this protein as the product of the ST gene, an extract of labelled proteins prepared by *in vitro* transcription of pRIT 10220 was analysed for the presence of material reacting with antibody raised against purified ST. Proteins binding non-specifically to antibodies were first removed from the extract by sequential adsorption to pre-immune serum and Sepharose-coupled protein A from *Staphylococcus aureus*. The extract was subsequently incubated with immune serum and the specific antibody-antigen complexes so-formed examined by electrophoresis in the presence of SDS. Figure 2b not only shows that

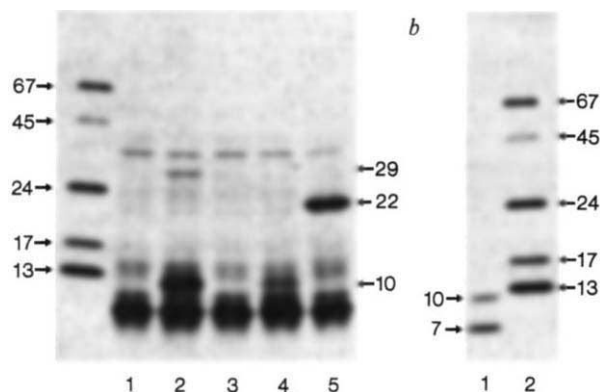


Fig. 2 *In vitro* synthesis and immunoprecipitation of plasmid-coded proteins. *a*, *In vitro* transcription-translation reactions were performed in the conditions described in ref. 21 in a final volume of 30 µl containing 2 µg of DNA and 50 µCi ³H-leucine. After 30 min at 37 °C the proteins were acid-precipitated, applied to an exponential 8–25% polyacrylamide gel using a standard buffer system²⁴ and subsequently visualised by fluorography²⁵. DNA samples were (1) no DNA, (2) pRIT 10220, (3) vector pBR322, (4) pRIT 10211, (5) vector pACYC184. Figures give the MW × 10³. The 29,000 MW protein present in slots 2 and 3 has not been rigorously identified; the 22,000 MW protein present in slot 5 but absent from 4 is probably the product of the gene encoding chloramphenicol resistance since the insertion into the *Eco*RI site of pACYC184 disrupts this gene¹⁹. *b*, pRIT 10220 DNA was transcribed and translated as described in (*a*). The sample was diluted to 200 µl in a final concentration of 0.5 M KCl, 100 mM Tris-HCl pH 8.8, 2% Triton X-100, 100 µg ml⁻¹ each of soybean trypsin inhibitor and phenylmethyl sulphonyl fluoride, and treated sequentially with 2 µl of pre-immune serum (1 h, 25 °C) and 5 mg protein A-Sepharose (Cl-4B, Pharmacia) (1 h, 25 °C). The supernatant after centrifugation was treated with 5 µl of immune serum (18 h, 4 °C) and 8 mg protein A-Sepharose (1 h, 25 °C). The Sepharose was collected by centrifugation, washed extensively, and bound proteins denatured and examined by SDS gel electrophoresis and fluorography as before. Slot 1 is the anti-ST immunoprecipitate and slot 2 contains marker proteins. The anti-ST immune serum was raised against modified purified toxin and has been shown to seroneutralise both purified preparations of ST and culture supernatants from ST producing bacteria in the baby mouse test. Preimmune sera from the same rabbits were ineffective in this test.

the 10,000 MW protein reacts with the anti-ST serum, but further reveals a second smaller protein (MW ~7,000) which also reacts with the serum.

The appearance of two proteins both of which react with the anti-ST serum suggests that the larger protein might be the primary translation product of the ST gene, and that the ST protein, in common with many other exported proteins²², would also be subject to specific proteolytic processing associated with trans-membrane transport. In support of this hypothesis are the findings that not only is the *in vitro* transcription/translation

Table 1 Biological activity of ST enterotoxin synthesised *in vivo* and *in vitro*

Source of toxin	No. of mice	Body weight (g)	Gut weight (g)	Ratio
<i>E. coli</i> C600/pRIT 10220 culture supernatant	4	7.415	0.826	0.1214
	4	6.985	0.881	
	4	6.949	0.885	
<i>E. coli</i> C600/pBR322 culture supernatant	4	6.901	0.367	0.0542
	4	6.195	0.351	
	4	6.651	0.352	
<i>In vitro</i> synthesis directed by pRIT 10220 DNA	4	7.023	0.869	0.1237
<i>In vitro</i> synthesis directed by pBR322 DNA	4	6.539	0.418	0.0639
<i>In vitro</i> synthesis mixture, no exogenous DNA	4	6.864	0.427	0.0622

One-day-old mice were injected intragastrically with 0.025 ml of culture supernatant of *E. coli* grown to saturation in Trypticase Soy Broth (Difco) medium or with 0.05 ml of *in vitro* transcription/translation mixture directed by 27 µg of plasmid pRIT 10220 DNA. The mice were killed after 2 h at 28 °C and the carcass and intestines separated and weighed. A gut/body ratio of 0.075 is considered weakly positive, a ratio of 0.100 or above strongly positive.

system partially active in the processing of another exported polypeptide, the penicillinase from *Bacillus licheniformis*²³ but also the published MW of the purified toxin (5,000)¹³ is, within experimental error, in accord with that estimated for the smaller protein (MW 7,000) described here.

By comparison with the relative intensities of the marker proteins in Fig. 2, we were able to estimate that 2 µg of plasmid pRIT 10220 was capable of directing the synthesis of some 0.5–5 ng of toxin in our standard assay conditions. This result encouraged the scaling up of the reaction and direct biological assay of the products of synthesis by intragastric injection of infant mice. The results shown in Table 1 demonstrate that the incubation of cloned ST-DNA in a coupled RNA- and protein-synthesising system results in the appearance of biologically active heat-stable enterotoxin.

We thank Drs L. Dobrescu and F. Van Wijnendaele for rabbit anti-ST antiserum and Miss M. Peeters for performing the infant mice assays. These investigations were supported in part by a grant to Smith Kline-RIT from IRSIA (Belgium).

Received 16 October 1979; accepted 12 February 1980.

- Sack, R. B. *A. Rev. Microbiol.* **29**, 333–353 (1975).
- Gyles, C. L. *Ann. N.Y. Acad. Sci.* **176**, 314–322 (1971).
- Smith, N. & Sack, R. B. *J. infect. Dis.* **127**, 164–170 (1973).
- Dallas, W. S. & Falkow, S. *Nature* **277**, 406–407 (1979).
- Evans, D. J., Chen, L. C., Curlin, G. T. & Evans, D. G. *Nature new Biol.* **236**, 137–138 (1972).
- Dean, A. G., Ching, Y.-C., Williams, R. C. & Harden, L. B. *J. infect. Dis.* **125**, 407–411 (1972).
- Sivaswamy, G. & Gyles, C. L. *Can. J. comp. Med.* **40**, 241–246 (1976).
- Kaekenbeek, A., Schoenaers, F., Pastoret, P. P. & Josse, M. *Ann. Med. Vet.* **121**, 55–57 (1977).
- Ryder, H. W. *et al. New Engl. J. Med.* **295**, 849–853 (1976).
- Kudoh, Y., Zen-Youji, H., Matsushita, S., Sakai, S. & Maruyama, T. *Microbiol. Immun.* **21**, 175–178 (1977).
- Hughes, J. M., Murad, F., Chang, B. & Guerrant, R. L. *Nature* **271**, 755–756 (1978).
- Field, M., Graf, L. H., Laird, W. J. & Smith, P. L. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2800–2804 (1978).
- Alderete, J. F. & Robertson, D. C. *Infect. Immun.* **19**, 1021–1030 (1978).
- Smith, H. W. & Halls, S. *J. gen. Microbiol.* **52**, 319–334 (1968).
- Gyles, C. L., So, M. & Falkow, S. *J. infect. Dis.* **130**, 40–49 (1974).
- Smith, H. W. *Soc. appl. Bact. Symp.* **227**, 227–242 (1976).
- So, M., Heffron, F. & McCarthy, B. *J. Nature* **277**, 453–456 (1979).
- Harford, N. *et al.* (in preparation).
- Chang, A. C. Y. & Cohen, S. N. *J. Bact.* **134**, 1141–1156 (1978).
- Bolivar, F. *et al. Gene* **2**, 95–113 (1977).
- Fuchs, E. *Eur. J. Biochem.* **63**, 15–22 (1976).
- Blöbel, G. & Dobberstein, B. *J. Cell. Biol.* **67**, 835–851 (1975).
- Sarvas, M. *et al.* (in preparation).
- Laemmli, U. K. *Nature* **227**, 680–685 (1970).
- Laskey, R. A. & Mills, A. D. *Eur. J. Biochem.* **56**, 335–341 (1975).

Vegetative *Dictyostelium* cells containing 17 actin genes express a single major actin

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Although actin is highly conserved between different eukaryotic species¹⁻³, six tissue-specific actins have been characterised in higher vertebrates by complete amino acid sequence analysis (two cytoplasmic actins, two smooth muscle actins and two sarcomeric actins)⁴⁻⁸. Their tissue specificity suggests they may differ in some important although unknown physiological property. Actin expression in lower eukaryotes seems to be a simpler process than in higher eukaryotes since biochemical experiments have indicated only one major type in purified preparations from various species^{1-3,9-12}. However, Firtel *et al.* have isolated several recombinant plasmids containing sequences of *Dictyostelium discoideum* DNA complementary to actin messenger RNA¹³⁻¹⁵ and have suggested that this unicellular slime mould may have 17 actin genes^{15,16} potentially giving rise to several different actins. We have, therefore, determined the complete amino acid sequence of actin from vegetative *Dictyostelium* cells. This sequence is unique and agrees with the DNA sequences of four actin genes for that region of the DNA, which is currently known. The protein sequence does not agree with the three other 'genes' and we discuss the possible expression of minor actin species.

Figure 1 shows the primary structure of actin purified¹¹ from vegetative cells determined by procedures used to study amino acid sequence homologies in actins^{5,6}. Comparison with the sequence of actin from *Physarum polycephalum*³ shows that the two proteins differ by only four exchanges. Three of these involve conservative replacements of uncharged amino acids in positions 189, 228 and 313, and the fourth at position 1 involves a charged residue; here *Dictyostelium* actin has aspartate whereas *Physarum* actin has glutamate. Comparison of the sequences of the two lower eukaryotic actins to those of the mammalian cytoplasmic actins⁶ shows that substitutions are not

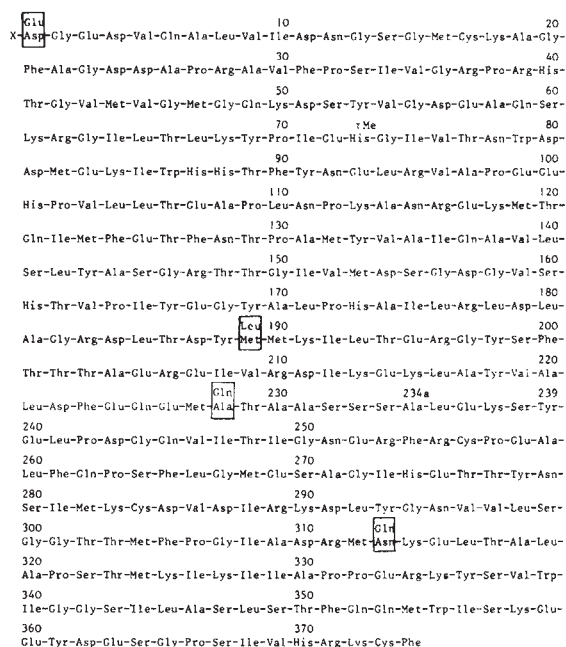


Fig. 1 The amino acid sequence of actin purified from vegetative cells of *Dictyostelium discoideum*. The order of the proteolytic fragments in the polypeptide chain is based on the homology with the known sequence of rabbit skeletal muscle actin¹⁷ taking into account the minor revisions proposed subsequently^{5,6,18}. An extra residue following residue 234 has been found in all actins so far studied and has been most likely overlooked in the original rabbit skeletal muscle actin sequence. To avoid renumbering of the previously documented amino acid exchanges past this residue we follow the proposal¹⁸ to indicate this residue as 234a. X is the acetyl blocking group¹² typical for all actins and His^{7Me} is N⁷-methyl-histidine. Residues shown in the top line of the boxes indicate the corresponding amino acid residues in the actin from *Physarum polycephalum*³.

distributed randomly, but are concentrated in residues 1 to 6 and occur occasionally past residue 159. Residues 7 to 159 seem conserved, as only one change has been detected. The same region of mammalian skeletal muscle actin differs from cytoplasmic actins by 7 replacements⁶, suggesting perhaps functional importance of this part for non-muscle actins.

Table 1 summarises the codons at the 5'-end of the *Dictyostelium* actin genes which could give rise to amino acid

Table 1 Summary of DNA sequence data for seven actin genes (from ref. 16) compared to the major *D. discoideum* actin (see Fig. 1)

Positions in amino acid sequence	0	1	2	3	12	15	16	17	30	34	38	40
Genes	Codons of DNA sequence of actin genes											
pDds actin B ₁	AUG	GAC	GGU	GAA	AAU	GGU	AUG	UGU	GUU	AUU	CCA	CAC
pDd actin 2-Sub 1	AUG	GAU	GGU	GAA	AAC	GGU	AUG	UGU	GUU	AUU	CCA	CAU
pDd actin 7	AUG	GAC	GGU	GAA	AAU	GGU	AUG	UGU	GUU	AUU	CCA	CAU
pDd actin 5	AUG	GAC	GGU	GAA	AAC	GGU	AUG	UGU	GUU	AUU	CCA	CAC
Corresponding amino acid in actin of <i>Dictyostelium</i>		Asp	Gly	Glu	Asn	Gly	Met	Cys	Val	Ile	Pro	His
pDd actin 3	AUG	GAA	AGU	GAA	AAU	GGU	AUG	UGU	CUU	AUU	CCU	UAU
M6	AUG	GAC	GGU	GAA	AAU	GGA	AUG	UGU	CUU	AUU	CCU	UAU
									Leu			Tyr
pDd actin 2-Sub 2	AUG	GAA	UGU	GGA	AAA	AGU	AUA	AGU	GUU	AAU	CAA	UAU
		Glu	Cys	Gly	Lys	Ser	Ile	Ser		Asn	Gln	Tyr

Only those codons are given where the presumptive actin gene products give rise to different amino acid residues. Nomenclature for actin genes is taken from ref. 16. The amino acid differences predicted by genes pDd actin 3, M6 and pDd actin 2-Sub 2 are indicated. The methionine residue at position 0 is due to initiation of protein synthesis and removed post-translationally before acetylation of the aspartic acid at position 1 (reviewed in ref. 19).

differences in the N-terminal part of the actin polypeptides (data taken from Firtel *et al.*¹⁶). On comparison with the amino acid sequence (Fig. 1) three different gene types can be distinguished. The first includes pDd actin 2-Sub 1, 5, 7, and pcDd actin B₁, the only clone synthesised from poly(A) containing mRNA. Although these four genes show subtle nucleotide differences they code for the same protein sequence, which agrees fully with the actin sequence given above. In the case of gene pDd actin 7 a complete correlation for the first 67 amino acids is possible. Provided further analysis does not reveal differences between DNA and protein sequence, the genes could all be true actin genes coding, either simultaneously or at different times of the cell and/or differentiation cycle, for the major cellular actin. The second gene type gives rise to actins differing from the major actin in only a few positions; out of the 50 residues currently covered by DNA sequence; pDd 3 actin differs in four and M6 actin in two amino acids (discussed below). Gene pDd actin 2-Sub 2 provides the third gene type. It comprises a putative actin with a high number of exchanges: that is, 10 for the first 40 residues predicted by the current DNA data (Fig. 1, Table 1). Although this divergence (25%) is only slightly higher than that observed in the same region between mammalian skeletal muscle actin and mammalian β -cytoplasmic actin (20%)^{4,6}, a change in the alignment of clustered constant residues typical of the N-terminal regions of all eukaryotic actins^{3,5} so far studied is predicted (note position of cysteine and lysine residues). Therefore if pDd 2-Sub 2 actin is expressed at all, it may be involved in special functions.

Are genes pDd 3 and M6 silent or are they active at a low level or only at specific times during cellular differentiation? Some of the amino acid differences predicted by the DNA sequence would give rise to charge differences in the resulting actins. Several arguments indicate that the two predicted actins could only be expressed at less than 5% of the level of the major actin. First, the tryptic peptide containing at position 30 the presumptive valine to leucine change of M6 and pDd 3 actin (Table 1), would co-migrate in the fingerprint system with the corresponding peptide of the major actin. As the latter peptide does not contain leucine, it is possible to quantify the amount of M6 and pDd 3 actins by the amount of leucine detectable. Amino acid analysis indicates that M6 and pDd 3 actin, if present at all, can maximally account for 5% of the major actin type. Second, the histidine to tyrosine exchange at position 40 (Table 1) would alter the isoelectric point of pDd 3 and M6 actins. This change would also produce an additional more acidic peptide covering residues 40–50. Since such changes were not found, it can be concluded that if genes pDd 3 and M6 are expressed, the corresponding actins can account together for maximally 10% of the final purified actin. Also, because the purification procedure isolates only ~30% of the cellular actin¹¹, the possibility that certain actin forms were lost preferentially cannot be excluded. To avoid this problem we used a method to characterise actins by the identification of the N-terminal tryptic peptide (residues 1–18). This very acidic peptide can be specifically isolated from a tryptic digest of ¹⁴C-carboxymethylated total cellular protein and then characterised in a two-dimensional paper electrophoresis system⁴. Figure 2 shows the *Dictyostelium* actin N-terminal tryptic peptide in this system together with the corresponding peptides from five different bovine actins and *Physarum* actin. The results show that vegetative *Dictyostelium* cells express only one actin (Fig. 2C). Its N-terminal peptide migrates slightly more acidically than the corresponding peptide of *Physarum*, in agreement with the aspartate to glutamate exchange at position 1 (see above). There is no evidence for actin types with N-terminal peptides different from the one predicted by the first type of genes (see Table 1). The N-terminal peptide expected from pDd 3 actin is clearly absent. This peptide should migrate like the *Physarum* peptide as it differs only at position 2 by a serine to glycine change. From the ratios of label present in the spot of the *Dictyostelium* actin peptide and the area in the map corresponding to the position of the presumptive pDd 3 actin derived peptide, the latter actin

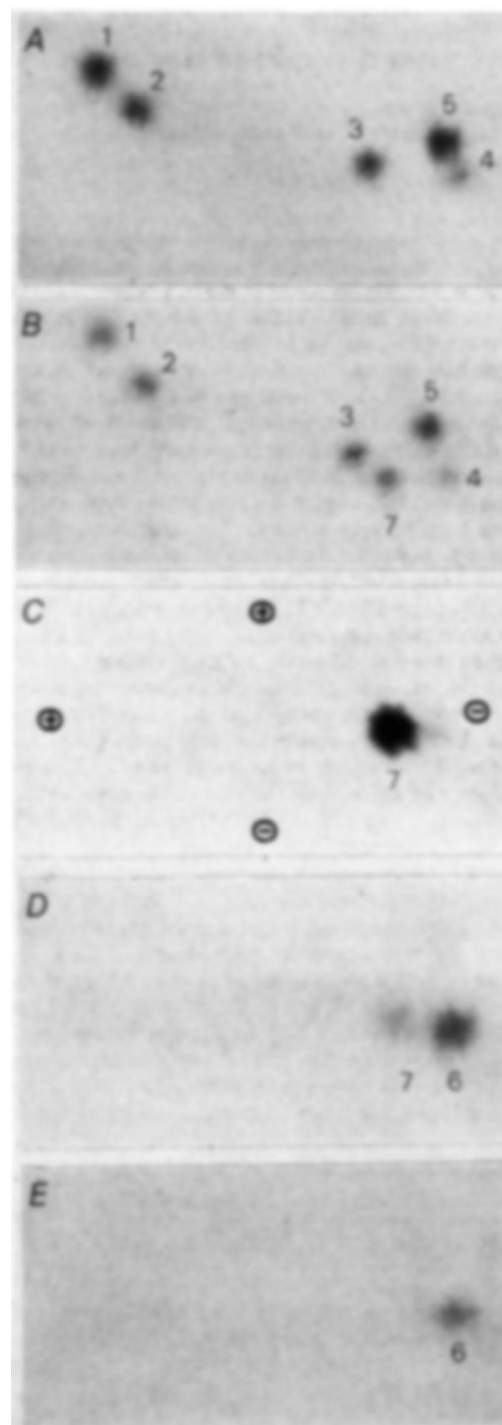


Fig. 2 Two-dimensional separation pattern of the ¹⁴C-carboxymethylated actin N-terminal tryptic peptide from a complete lysate of vegetative cells of *Dictyostelium* (spot 7). This peptide is compared with the corresponding peptides isolated from the following bovine actins: α -like smooth muscle actin of aorta (spot 1), γ -like smooth muscle actin of the rumen (spot 2), β -non-muscle actin (spot 3), γ -non-muscle actin (spot 4), α -skeletal muscle actin (spot 5) and the corresponding peptide from *Physarum polycephalum* (spot 6). A, bovine actins (spots 1–5); B, bovine actins (spots 1–5) + vegetative *Dictyostelium* actin (spot 7); C, *Dictyostelium* actin (spot 7); D, *Dictyostelium* actin (spot 7) + *Physarum* actin (spot 6); E, *Physarum* actin (spot 6). Horizontal separation is by electrophoresis at pH 3.3 and vertical separation is by electrophoresis at pH 6.5. For methods see refs 4 and 5.

could maximally be present at less than 6% of the total actin of vegetative cells.

Thus our studies have not detected minor actin species in addition to the unique actin type for which we provide the complete amino acid sequence. This sequence is compatible with the DNA sequences of four actin genes but does not accommodate the other three currently described. It remains possible that the other genes code for actins present either at very low level in vegetative cells or expressed only at specific times during cellular differentiation, particularly since different isoelectric forms of actin have been reported in one¹⁵, but not a second¹², *in vitro* translation study. The methods described here should provide answers to these questions when cells at different developmental stages and actins translated *in vitro* are used.

We thank Dr J. Spudich for a gift of purified *Dictyostelium* actin, M. Puype for technical assistance and Drs M. Osborn and A. Bretscher for discussions. J.V. is bevoegdverklaard navorser at the Belgian National Fund of Scientific Research and thanks the NFWO for a grant.

Note added in proof: We have now shown that preaggregation cells of *Dictyostelium* also show the same N-terminal peptide as vegetative cells.

Received 30 November 1979; accepted 13 February 1980.

1. Korn, E. D. *Proc. natn. Acad. Sci. U.S.A.* **75**, 588–599 (1978).
2. Elzinga, M. & Lu, R. C. in *Contractile Systems in Non-Muscle Tissues* (eds Perry, S. V., Margreth, A. & Adelstein, R.) 29–37 (Elsevier, Amsterdam, 1976).
3. Vandekerckhove, J. & Weber, K. *Nature* **276**, 720–721 (1978).
4. Vandekerckhove, J. & Weber, K. *Differentiation* **14**, 123–133 (1979).
5. Vandekerckhove, J. & Weber, K. *J. molec. Biol.* **126**, 782–802 (1978).
6. Vandekerckhove, J. & Weber, K. *Eur. J. Biochem.* **90**, 451–462 (1978).
7. Vandekerckhove, J. & Weber, K. *Proc. natn. Acad. Sci. U.S.A.* **75**, 1106–1110.
8. Vandekerckhove, J. & Weber, K. *FEBS Lett.* **102**, 219–222 (1979).
9. Gordon, D. J., Boyer, J. K. & Korn, E. D. *J. biol. Chem.* **252**, 8300–8309 (1977).
10. Zechel, K. & Weber, K. *Eur. J. Biochem.* **89**, 105–112 (1978).
11. Uyemura, D. G., Brown, S. S. & Spudich, J. A. *J. biol. Chem.* **253**, 9088–9096 (1978).
12. Rubenstein, P. & Deuchler, J. *J. biol. Chem.* **254**, 11142–11147 (1979).
13. Bender, W. *et al. Cell* **15**, 779–788 (1978).
14. McKeown, M. *et al. Cell* **15**, 789–800 (1978).
15. Kindle, K. & Firtel, R. A. *Cell* **15**, 763–778 (1978).
16. Firtel, R. A., Timm, R., Kimmel, A. R. & McKeown, M. *Proc. natn. Acad. Sci. U.S.A.* **76**, 6206–6210 (1979).
17. Collins, J. H. & Elzinga, M. *J. biol. Chem.* **250**, 5915–5920 (1975).
18. Lu, R. C. & Elzinga, M. *Biochemistry* **16**, 5801–5806 (1977).
19. Revel, M. in *Molecular Mechanisms of Protein Biosynthesis* (eds Weissbach, H. & Pestka, S.) 246–322 (Academic, New York, 1977).

Long spacers among ribosomal genes of *Drosophila melanogaster*

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The fruit fly, *Drosophila melanogaster*, has 200 tandemly arranged copies of the ribosomal RNA genes (rDNA) per haploid genome^{1,2}. One such cluster of rRNA genes occurs on the X and one on the Y chromosome. The basic repeating unit of the rRNA gene consists of a segment coding for 18S rRNA and 28S rRNA followed by a non-transcribed spacer³ (Fig. 1). In the X chromosome, there are two major size classes (12 and 17 kilobases) and numerous minor size classes of rDNA repeats^{3–7}. Most of this length heterogeneity is generated by insertions at a specific site in the 28S gene^{3–7}. The frequency and size patterns of these insertions in the 28S gene differ in the X and Y chromosomes^{4,7,8}. Electron microscopic analysis of rDNA-rRNA hybrids has shown that there is also length heterogeneity in the rDNA non-transcribed spacer^{4,6,7}. This heterogeneity is due in part to internal sequence repetition^{7,9}. We have now examined further the length heterogeneity of the rDNA spacer and have observed a class of spacers that we shall refer to as 'long spacers'. The size and frequency of these long spacers are different in the X and Y rDNA.

The resistance of the rDNA spacer to digestion with the endonuclease *Hae*III (ref. 10) provides a convenient method for the analysis of rDNA spacer size. To determine the spectrum of spacer sizes on the X and Y chromosomes, DNA from X/X or *sc*⁴-*sc*⁸/Y flies was digested with *Hae*III. (*sc*⁴-*sc*⁸ is an X chromosome that is at least 90% deficient in rDNA¹¹.) The resulting fragments were separated by agarose gel electrophoresis and subsequently transferred to nitrocellulose filters by the method of Wahl *et al.*¹², a modification of the Southern technique¹³, which increases the efficiency of transfer of large DNA fragments. The filter-bound fragments were hybridised to ¹²⁵I-labelled spacer DNA¹⁴ and finally visualised by autoradiography. The results of this experiment are shown in Fig. 2. In addition to the expected bands at 4–7 kilobases⁶, fragments at 11, 14 and 20 kilobases are apparent. We shall refer to fragments longer than 9 kilobases as long spacer. To insure that these long spacer fragments were not a result of underdigestion with *Hae*III, the DNAs were redigested with 20-fold excess of enzyme. These experiments yielded patterns identical to those shown in Fig. 2. Digestion with *Hae*III of other samples of X/X and *sc*⁴-*sc*⁸/Y DNAs prepared from independently derived stocks also yielded patterns identical to those in Fig. 2.

To show that the long spacers are continuous with the rRNA structural gene rather than surrounded by other sequences, DNA restricted with *Hae*III was probed with ¹²⁵I-labelled rRNA. In this case, the autoradiographic signal arises from the small segment of 28S rDNA which remains attached to spacer after restriction with *Hae*III (see Fig. 1). Although the intensity of signal of the long spacer fragments was weaker than that observed using ¹²⁵I-labelled spacer DNA as probe, patterns almost identical to those shown in Fig. 2 were obtained. Thus, the pieces of DNA containing the long spacers also contain rDNA genes.

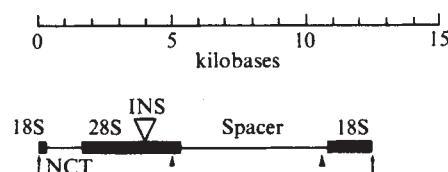


Fig. 1 Repeating unit structure of the ribosomal RNA gene in *Drosophila melanogaster*. The ribosomal gene consists of regions coding for 18S rRNA, 28S rRNA, a non-conserved transcribed (NCT) sequence and a non-transcribed spacer. Length heterogeneity occurs in the insertion (INS) in the 28S rRNA and in spacer sequences. The sites recognised by the restriction enzymes *Eco*RI and *Hae*III are indicated by arrows (↑) and arrowheads (▲), respectively. The map for *Hae*III is incomplete; however, there are no additional sites between the two sites shown¹⁰.

As shown in Fig. 2, digestion with *Hae*III generates different spacer fragment patterns for DNAs derived from X or Y DNA. In addition to the major bands at 5–6 kilobases, there are bands at 20, 11, 7.8 and 7.4 kilobases on DNA from the X chromosome. In DNA from the Y chromosome, bands appear at 14, 11, 5.7 and 5 kilobases and minor bands at 8.9, 7.6 and 6.4 kilobases. Densitometer scans indicate that most (~85%) of the spacer DNA from the X chromosome is 5.7 ± 1.9 kilobases long and that 9.8% makes up the 7.8- and 11-kilobase bands. These results agree with the data of Pellegrini *et al.*⁶, who found ~10% of spacer sequences between 7.5 and 11.5 kilobases and the remainder at 5.67 ± 1.92 kilobases. Their electron microscopic methods, however, would not have detected the 14- and 20-kilobase segments. Approximately 80% of the spacer from the Y chromosome is between 4 and 7.6 kilobases. As there are 200 rRNA genes per nucleolus² and assuming that each is linked to a spacer 5.7 ± 1.9 kilobases long, we estimate from the densitometer scans that there are about three copies of the 20-kilobase spacer fragment and five copies of the 11-kilobase fragment in the X chromosome nucleolus organiser. Similarly,

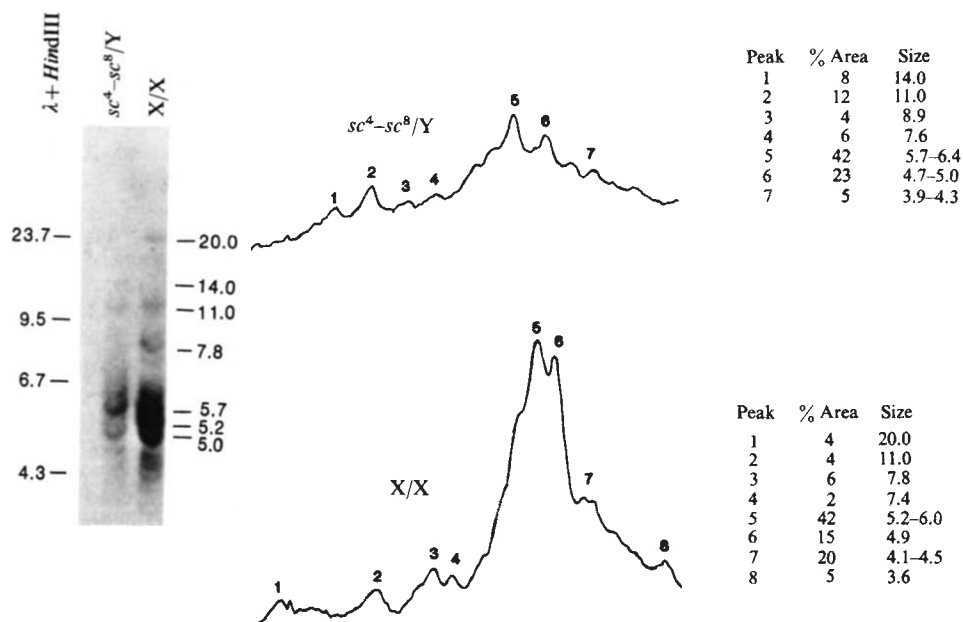


Fig. 2 *HaeIII* restriction patterns of rDNA spacer in *Drosophila melanogaster*. DNA (2 µg) from *sc*⁴-*sc*⁸/Y male and ORE-R X/X female flies was digested with *HaeIII*, separated on the same 0.7% agarose gel, transferred to a single nitrocellulose filter and hybridised to ¹²⁵I-labelled spacer (specific activity 4 × 10⁷ c.p.m. per µg) DNA and a 200-fold excess of purified unlabelled rRNA. (*sc*⁴-*sc*⁸ designates an X chromosome deficient in at least 90% of its rDNA¹¹.) The size in kilobases of bands from X and Y derived DNA is given on the right. The size of the large fragments was confirmed by electrophoresis through 0.4% agarose. Densitometer scans of the X/X and *sc*⁴-*sc*⁸/Y lanes and the area under each peak are shown on the far right.

we calculate that there are about 8 copies of the 14-kilobase spacer fragment, and 16 copies of the 11-kilobase fragment in the Y chromosome rDNA cluster.

It is possible that the fragments which hybridise to spacer segments do so because they share homology with the spacer but are not actually part of the structure of rDNA. For example, they might cross-hybridise with centromeric satellite sequences. If so, *in situ* hybridisation of ¹²⁵I-labelled spacer to polytene chromosomes should detect the presence of such sequences elsewhere in the genome. As shown in Fig. 3, the ¹²⁵I-labelled spacer hybridises exclusively to the nucleolus. Even extreme over-exposure (15 d) revealed no grains consistently over any portion of the genome other than the nucleolus. Thus, most, if not all, of the spacer sequences, including the long spacer fragments, are part of the structure of the rRNA genes and are present in the nucleolus.

Electron microscopy and restriction enzyme analysis of cloned and uncloned rDNA repeat units have shown that the non-transcribed spacer is moderately heterogeneous in length (5.7 ± 1.9 kilobases)^{4,6,7} and probably consists mainly of repeated sequence elements^{7,9}. We have shown that spacers longer than 9 kilobases also occur in ribosomal DNA. The organisation of the long spacers has not been further examined and it is possible that in addition to spacer sequences, non-spacer elements such as insertion sequences also reside in these gene units. Although the sequences of the major class of spacers are very similar in the X and Y chromosomes⁷, our results indicate that there are clear size and frequency differences between the long spacers derived from the X and Y chromosomes.

The heterogeneity observed in the size and frequency of long spacer supports the contention of Tartof and Dawid⁸ that the rDNA genes on the X and Y chromosomes are maintained separately without genetic exchange. Two previous pieces of evidence also support this argument. Wellauer *et al.*⁷ and Tartof and Dawid⁸ have shown that type 1 (5 kilobase) inserts which are frequent in X rDNA are absent in Y rDNA, and Yagura *et al.*¹⁵ have recently demonstrated that the primary structure of 18S rRNA transcribed from X rDNA differs from that of Y rDNA. In the light of these observations, it seems unlikely that genetic exchange is responsible for the similarity of transcribed^{7,16} and spacer⁷ sequences. Selection pressures may instead have a major role in the parallel evolution of tandemly repeated genes in non-homologous chromosomes.

The presence of a small number of copies of long spacer sequences within the rDNA suggests that the rRNA genes are not organised as a simple tandem array of 200 repeating units. Rather, they may be grouped into subclusters separated from



Fig. 3 *In situ* hybridisation of ¹²⁵I-labelled spacer to *D. melanogaster* polytene chromosomes. As noted in these representative nuclei, all grains appear over the nucleolus (exposure, 3 days).

each other by long spacer. Note that changes in rRNA gene number arising from rDNA magnification or reduction tend to occur as single stepwise increases or decreases of approximately 50 or 100 rRNA genes^{2,17}. On the basis of several experimental lines of evidence, it was initially proposed that rDNA magnification and reduction are driven by unequal sister chromatid exchange¹⁸. It is conceivable that such exchanges may occur at sites in long spacer DNA.

This work was supported by grants GM-19194, CA-06927, RR-05539 and CA-09035 awarded to the Institute for Cancer Research and by an appropriation from the Commonwealth of Pennsylvania.

Received 10 December 1979; accepted 6 February 1980.

- Ritossa, F. M., Atwood, K. C., Lindsley, D. L. & Spiegelman, S. *Nat. Cancer Inst. Monogr.* **23**, 449–472 (1966).
- Tartof, K. D. *Science* **171**, 294–297 (1971).
- Glover, D. M. & Hogness, D. S. *Cell* **10**, 167–176 (1977).
- Wellauer, P. K. & Dawid, I. B. *Cell* **10**, 193–212 (1977).
- White, R. L. & Hogness, D. S. *Cell* **10**, 177–192 (1977).
- Pellegrini, M., Manning, J. & Davidson, N. *Cell* **10**, 213–224 (1977).
- Wellauer, P. K., Dawid, I. B. & Tartof, K. D. *Cell* **14**, 269–278 (1978).
- Tartof, K. D. & Dawid, I. B. *Nature* **263**, 27–30 (1976).
- Long, E. O. & Dawid, I. B. *Nucleic Acids Res.* **7**, 205–215 (1979).
- Dawid, I. B. & Botchan, P. *Proc. natn. Acad. Sci. U.S.A.* **74**, 4233–4237 (1977).
- Tartof, K. D. *Cold Spring Harb. Symp. quant. Biol.* **38**, 491–500 (1973).
- Wahl, G. M., Stern, M. & Stark, G. R. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3683–3687 (1979).
- Southern, E. M. *J. molec. Biol.* **98**, 503–517 (1975).
- Tartof, K. D. *Cell* **17**, 607–614 (1979).
- Yagura, T., Yagura, M. & Muramatsu, M. *J. molec. Biol.* **133**, 533–547 (1979).
- Maden, B. E. H. & Tartof, K. D. *J. molec. Biol.* **90**, 51–64 (1974).
- Tartof, K. D. *Genetics* **73**, 57–71 (1973).
- Tartof, K. D. *Proc. natn. Acad. Sci. U.S.A.* **71**, 1272–1276 (1974).

A *trans*-acting factor mediates inversion of a specific DNA segment in flagellar phase variation of *Salmonella*

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Inversions of specific DNA segments have been shown to be involved in regulating gene expression in several systems, and flagellar phase variation in *Salmonella* is one example. Strains of diphasic *Salmonella* species, such as *S. typhimurium*, possess two non-allelic structural genes, *H1* and *H2*, for flagellin, the component protein of flagellar filaments. One or the other is expressed in a bacterial clone. Lederberg and Iino¹ reported that the switch from the expression of one gene to that of the other was controlled by a genetic element linked to the *H2* gene and that the state of *H2* determined the flagellar phase of the bacterium. Simon *et al.*^{2–4} have shown that the expression of *H2* is controlled by a recombination which inverts a region of DNA containing an element necessary for the transcription of *H2*. We report here that prophages P1 and Mu produce a *trans*-acting cytoplasmic factor which mediates the inversion of a specific DNA segment in flagellar phase variation of *Salmonella*.

H1 and *H2* specify phase-1 and phase-2 flagellin, respectively¹. Genetic^{5–7} and biochemical⁸ studies have shown that *H2* constitutes an operon with *rh1* which specifies a repressor of the expression of *H1*. When the *H2*-operon is active (*H2*-on), phase-2 flagellin is synthesised and *H1* is repressed by the product of *rh1*. When the *H2*-operon is inactive (*H2*-off), *H2* and *rh1* are not transcribed and, thus, phase-1 flagellin is synthesised. The element which determines the state of the *H2*-operon is termed phase determinant (*PD*)⁹. It is adjacent to the *H2*-operon and regulates its activity by inversion^{2–4}. This invertible segment consists of 800 base pairs of DNA². From genetic studies of a strain of *Salmonella abortusequi*, a species

Table 1 Effect of the exogenous *vh2*⁺ allele on the flagellar phase variation of the *vh2*[−] strain

Plasmids	Swarm-forming ability on motility agar plates containing anti-flagella antisera			
	none	e, n, x	a	e, n, x + a
None	+	−	+	−
pCR1	+	−	+	−
pKK1200	+	+	+	−

The bacterial strain was KK1027, a *strA recA* derivative of SL23 (Fig. 1). The hybrid plasmid, pKK1200, contained a 3.75-kb DNA fragment of *Salmonella* inserted into the *EcoRI* site of pCR1 (ref. 19). This fragment containing the *vh2*⁺ allele, *PD*, and *H2* but not *rh1* was derived from pJZ200 (ref. 20). This *H2* gene specified e, n, x-flagellin. Plasmids were introduced into KK1027 by F factor-mediated transfer from *E. coli* cells harbouring F' factor and either pCR1 or pKK1200. Motility agar plates contained, per litre of distilled water: 7.5 g trypton (Difco); 2.5 g NaCl (Nakarai) 2.5 g agar (Shoei). Antisera were prepared from rabbits immunised by flagella. For the motility tests, cells were inoculated on to motility agar plates and incubated at 37 °C for 1 or 2 d. +, Motile; −, non-motile.

that changes phase at very low frequency, Iino¹⁰ showed that the *vh2* gene controls the change of state of *H2*, to which it is closely linked. Diphasic strains carry the *vh2*⁺ allele and change their flagellar phase at frequencies of 10^{−3} to 10^{−5} per bacterial division¹¹. Stable-phase strains carry the *vh2*[−] allele and change their flagellar phase at frequencies lower than 10^{−7} per bacterial division¹⁰. Thus replacement of *vh2*⁺ by *vh2*[−] results in stabilisation of the *H2*-operon in whatever state it is at the time of the replacement. A model for flagellar phase variation of *Salmonella* is summarised in Fig. 1.

Among the factors involved in regulating flagellar phase variation of *Salmonella*, *PD* acts *cis* to the *H2*-operon^{2–4}. To determine whether the *vh2* gene acts in *cis* (and is therefore probably identical with *PD*) or in *trans*, a hybrid plasmid (pKK1200) containing the *vh2*⁺ allele was introduced into the *vh2*[−] strain (KK1027). This strain was a *strA recA* derivative of the *S. abortusequi* strain SL23 (Fig. 1, ref. 8). It was fixed in phase-2 by the *vh2*[−] allele and produced flagella antigenically characterised by the antigen e, n, x. The repressed phase-1 antigen type was a. As Table 1 shows, strain KK1027 carrying pKK1200 showed phase variation, producing some sub-clones with antigen e, n, x and others with antigen a. By contrast KK1027 carrying pCR1 or no plasmid failed to change its flagellar phase. The expression of the previously repressed chromosomal *H1* gene specifying antigen a shows that there is no *rh1* product in the cells concerned, and therefore that not only the plasmid-borne but also the chromosomal *H2*-operon is 'off'. Thus the plasmid-borne *vh2*⁺ gene seems to act in *trans*, to

Table 2 Effects of prophage P1 or Mu on flagellar phase variation of *vh2*[−] strains

Host strains	Plasmids	Production of motile cells
KK1251	None	−
	P1CMclr100	+
KK1252	None	−
	F'lac ⁺	−
	F'lacI ⁺ ::Mucls62	+

KK1251 and KK1252 were *galE* and *strA* derivatives of SJW1250 (Fig. 1), respectively. P1CMclr100 was a temperature-inducible mutant of P1 carrying a chloramphenicol-resistance element, Tn9 (ref. 21). Mucls62 was a temperature-inducible mutant of Mu-1 (ref. 18). P1CMclr100 lysogens were prepared from KK1251 according to Rosner²¹. (Prophage P1 persists in the plasmid state²².) F'lac⁺ or F'lacI⁺::Mucls62 factors were transferred, from *E. coli* strains harbouring either of them, to KK1252 according to Miller²³. For the motility tests, cells were inoculated on to motility agar plates and incubated at 30 °C for 5 h. One hundred clones were tested for each experiment. +, Presence of motile cells; −, absence of motile cells.

facilitate the change of state of *PD*, a change that is thought to involve inversion of the segment containing the promoter region of the *H2*-operon. This indicates that the *vh2* gene specifies a cytoplasmic factor that catalyses the inversion of *PD* in the flagellar phase variation of *Salmonella* (Fig. 1).

Bacteriophage Mu DNA contains a 3,000-base pair sequence—the G segment—that can undergo inversion¹², apparently correlated with the formation of infectious phage particles^{13,14}. A similar invertible region has been found in bacteriophage P1 DNA^{15,16} and termed the C region¹⁷. The G segment of Mu445-5 (ref. 18) defective in a *trans*-acting factor for G inversion has been shown to be inverted by the inversion system of P1 (ref. 14). We examined whether the inversion system of P1 (ref. 14). We examined whether the inversion systems of Mu or P1 could invert the *PD* of *Salmonella*.

Derivatives of a *vh2*⁻*H2*⁻ strain of *Salmonella* (SJW1250, Fig. 1) were chosen as tester strains. SJW1250 was fixed in phase-2 by the *vh2*⁻ allele and had no flagella because of a defect in the *H2* gene. Its repressed *H1* gene was intact and specified flagellin, characterised by antigen gt. If this strain changes its flagellar phase from *H2*-on to *H2*-off, gt-flagellin will be synthesised and, thus, the bacterium will become motile. Normally, it produces motile sub-clones at a frequency of 10⁻⁸. Using this system, we showed that the strains carrying P1 or Mu as prophage produced motile sub-clones at a high frequency.

Table 2 shows that KK1251 (a *galE* derivative of SJW1250) carrying P1Cmclr100 and KK1252 (an *strA* derivative of SJW1250) carrying F'lacI^s::Mucts62 were both able to form spreading colonies (swarms) on motility agar plates. They all produced gt-flagella, as revealed by slide agglutination tests with anti-gt antiserum. These results indicate that the change of flagellar phase from *H2*-on to *H2*-off occurred in culture. So do they also change flagellar phase from *H2*-off to *H2*-on and manifest phase variation? If their flagellar phase oscillates between *H2*-on (not flagellated) and *H2*-off (flagellated), cultures must contain both motile and non-motile bacteria. Therefore, clones cured of phase must be fixed in whatever state, either motile or non-motile, they are in at the time of curing. Table 3 shows that this was the case. Phages were reintroduced into some of the resulting non-motile clones to exclude the possibility that they were non-motile mutants rather than clones fixed in *H2*-on. The lysogens also changed their flagellar phase from non-motile to motile. Thus flagellar phase changed between *H2*-on and *H2*-off in the *vh2* strains carrying P1Cmclr100 or Mucts62. The rate of phase variation calculated from the results in Table 3 was higher than 10⁻² per bacterial division in both lysogens. This value was greater than that of *vh2*⁺ strains (for example, 10⁻³ per bacterial division in *S. typhimurium*¹¹). To exclude the possibility that the factor catalysing the inversion of *PD* might be produced by the chloramphenicol-resistance element (Tn9) of P1Cmclr100, temperature-resistant, P1-sensitive, and chloramphenicol-resistant clones were isolated from KK1251 carrying P1Cmclr100. They must have Tn9 inserted in their chromosomes and have been free from phages. The resulting

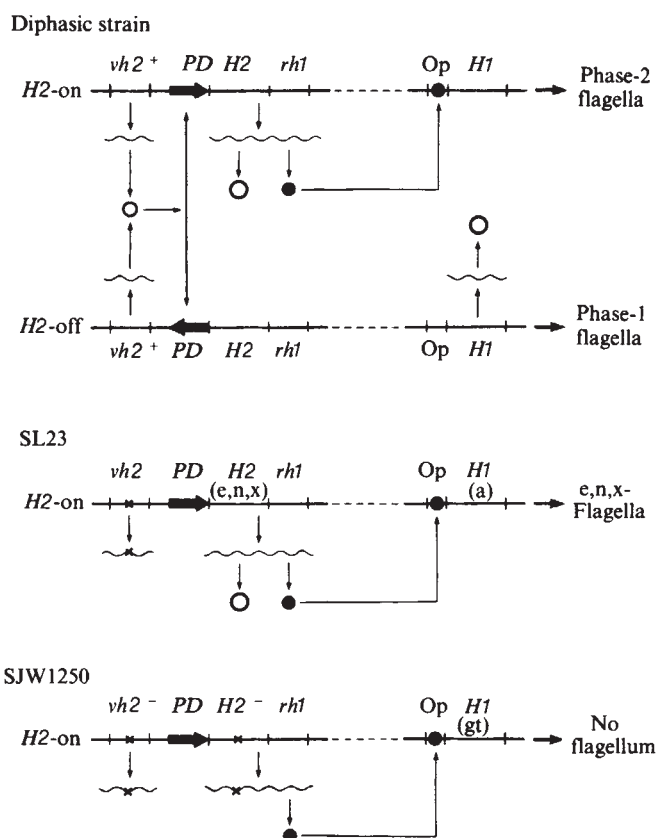


Fig. 1 Regulatory system for flagellar phase variation of *Salmonella*, diphasic strain, SL23 and SJW1250. *H1* and *H2* are structural genes for phase-1 and phase-2 flagellin, respectively. *H2* constitutes an operon with *rh1* which specifies the *H1*-repressor. *Op* indicates the binding site of the *H1*-repressor. *PD* indicates the phase determinant, which regulates the *H2* operon activity through a site-specific inversion. The *vh2* gene specifies a cytoplasmic factor which catalyses the site-specific inversion of *PD*. Horizontal heavy and wavy lines indicate the chromosomes of *Salmonella* and messenger RNA molecules, respectively. Large open and small closed circles represent flagellin and *H1*-repressor molecules, respectively. The small open circle represents the *vh2* gene product. Heavy arrows indicate the orientation of *PD*, which is expressed as the direction of transcription from a promoter residing in *PD*.

individual clones were also fixed in either the non-motile or motile state and did not change their flagellar phase (Table 3). Thus, P1 itself produces the factor catalysing the inversion of *PD*.

It is premature to conclude that the phase-specifying factor which catalyses the inversion of *PD* is also involved in the inversion of G segment in Mu or C region in P1. However, we have cloned the DNA fragment containing G segment of Mu or

Table 3 Flagellar phase of the *vh2*⁻ strains cured of prophage P1 or Mu

Host strains	Plasmids	Selected phenotypes	No. of clones tested	No. of clones fixed in		Frequency of phase variation
				motile	non-motile	
KK1251	P1Cmclr100	tr	400	70	330	2.8 × 10 ⁻²
		tr, Cm ^r	200	64	136	2.3 × 10 ⁻²
KK1252	F'lacI ^s ::Mucts62	tr	260	140	120	1.5 × 10 ⁻²

Motile clones harbouring either of the plasmids were grown in LB²³ at 30 °C for about 30 generations. Cultures of KK1251 harbouring P1Cmclr100 were diluted, plated on LB plates or LB plates containing 25 µg of chloramphenicol per ml, and incubated at 42 °C. The resulting isolates on the former plates were tested for P1 sensitivity and chloramphenicol resistance. All were P1-sensitive and chloramphenicol-sensitive and, thus, cured of P1Cmclr100. The resulting isolates on the latter plates were tested for P1 sensitivity, and all of them were found to be P1-sensitive. Thus, they carried Tn9 inserted on the chromosomes but no prophage. Cultures of KK1252 harbouring F'lacI^s::Mucts62 were diluted, plated on MacConkey lactose plates, and incubated at 42 °C. The resulting isolates were all found to form white colonies. For the motility tests, cells were inoculated on to motility agar plates and incubated at 42 °C for 5–24 h. Frequency of phase variation was expressed as the rate of change from motile (*H2*-off) to non-motile (*H2*-on) state per division of cells harbouring either prophage P1 or Mu. tr, Temperature-resistant; Cm^r, chloramphenicol-resistant.

C region of P1 on a plasmid vector, and KK1252 carrying the resulting hybrid plasmids also manifested phase variation (our manuscript in preparation). Thus it is highly plausible that such a factor also catalyses the inversion of G segment in Mu or C region in P1.

We propose that the genes whose products suppress the *vh2*⁻ allele of *Salmonella* should in general be termed *din*. In bacteriophage Mu, a gene which was thought to specify a *trans*-acting factor involved in G inversion was termed *gin*¹⁸. Because the *gin* gene was shown to be near the G segment¹⁸, the *din*⁺ activity of prophage Mu is probably specified by the *gin* gene. Enomoto and Stocker⁹ reported that when the *H2* locus of *Salmonella* together with the *vh2*⁻ allele was introduced into an

Escherichia coli strain by P1-mediated intergeneric transduction, the resulting transductants showed phase variation. This indicates that the *E. coli* strain also has a *din*⁺ activity, though *S. abortusequi* does not.

We thank Y. Komeda, S. Yamaguchi, M. Tsuda, and J. Roth for phages and bacterial strains, and M. Simon for a hybrid plasmid, pJZ200. All the experiments involving the construction and propagation of recombinant molecules were performed according to the 'Guidelines for Recombinant DNA experiments in Research Institutes such as Universities' (Ministry of Education, Science, and Culture, Japan, 31 March, 1979) in the P2 facility of our laboratory. This work was supported by a grant (410708) from MESC, Japan.

Received 26 November 1979; accepted 4 February 1980.

1. Lederberg, J. & Iino, T. *Genetics* **41**, 743-757 (1956).
2. Zieg, J., Silverman, M., Hilmen, M. & Simon, M. *Science* **196**, 170-172 (1977).
3. Zieg, J., Hilmen, M. & Simon, M. *Cell* **15**, 237-244 (1978).
4. Silverman, M., Zieg, J., Hilmen, M. & Simon, M. *Proc. natn. Acad. Sci. U.S.A.* **76**, 391-395 (1979).
5. Pearce, U. B. & Stocker, B. A. D. *J. gen. Microbiol.* **49**, 335-349 (1967).
6. Fujita, H., Yamaguchi, S. & Iino, T. *J. gen. Microbiol.* **76**, 127-134 (1973).
7. Silverman, M., Zieg, J. & Simon, M. *J. Bact.* **137**, 517-523 (1979).
8. Suzuki, H. & Iino, T. *J. molec. Biol.* **81**, 57-70 (1973).
9. Enomoto, M. & Stocker, B. A. D. *Genetics* **81**, 595-614 (1975).
10. Iino, T. *Genetics* **46**, 1465-1469 (1961).
11. Stocker, B. A. D. *J. Hyg., Camb.* **47**, 398-413 (1949).

12. Danielli, E., Abelson, J., Kim, J. S. & Davidson, N. *Virology* **51**, 237-239 (1973).
13. Bukhari, A. I. & Ambrosio, L. *Nature* **271**, 575-577 (1978).
14. Kamp, D., Kahmann, R., Zipser, D., Broker, T. R. & Chow, L. T. *Nature* **271**, 577-580 (1978).
15. Lee, H. J., Ohtsubo, E., Deonier, R. C. & Davidson, N. *J. molec. Biol.* **89**, 585-597 (1974).
16. Chow, L. T. & Bukhari, A. I. *Virology* **74**, 242-248 (1976).
17. Yun, T. & Vapnek, D. *Virology* **77**, 376-385 (1977).
18. Chow, L. T., Kahmann, R. & Kamp, D. *J. molec. Biol.* **113**, 591-609 (1977).
19. Covey, C., Richardson, D. & Carbon, J. *molec. gen. Genet.* **145**, 155-158 (1976).
20. Zieg, J., Silverman, M., Hilmen, M. & Simon, M. *Stadler Symp.* **10**, 65-76 (University of Missouri, Columbia, 1976).
21. Rosner, J. L. *Virology* **49**, 679-689 (1972).
22. Ikeda, H. & Tomizawa, J. *Cold Spring Harb. Symp. quant. Biol.* **33**, 791-798 (1968).
23. Miller, J. H. *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, New York, 1972).

Functional organisation of the photo-synthetic apparatus in heterocysts of nitrogen-fixing cyanobacteria

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Many cyanobacteria fix atmospheric N₂ only in specialised cells called heterocysts^{1,2}. Besides containing nitrogenase², heterocysts are distinguished biochemically from vegetative cells by their inability to evolve O₂ or to perform photosystem II activities^{1,3,4} and by a deficiency in the CO₂ fixing enzyme ribulose-1,5-bisphosphate carboxylase⁵. These cells also lack phycobiliproteins and phycobilisomes, that are part of the major light-collecting system for the light reactions of photosynthesis^{1,6}. The phycobilipigments, light-collecting chlorophyll *a* and the photochemical reaction centres constitute a photosynthetic unit (PSU)—the minimum number of pigments necessary to drive photochemistry, oxygen evolution and photosynthetic electron transport. Therefore, the PSU represents an energy generating unit. In cyanobacteria, photosynthesis, and hence the PSU, contributes both ATP and probably a strong reductant to drive nitrogen fixation⁸. Because of the distinct photosynthetic properties of these cells, especially in pigmentation, we have compared the functional organisation of the pigments and photochemical activity of the thylakoid membranes from vegetative and heterocyst cells of *Nostoc muscorum* and *Anabaena cylindrica*. We demonstrate that the PSU is modified during the differentiation of heterocysts to support the specialised energetic requirements of these nitrogen fixing cells.

Axenic cultures of *A. cylindrica* (Cambridge culture collection no. 1403/2a) and *N. muscorum* (strain 7119) were grown in nitrogen-free medium as described previously⁸. Heterocysts and vegetative cells were separated, purified and counted⁸. The purified heterocysts (Fig. 1) were ruptured at 16,000 p.s.i. in a French pressure cell. Membranes were solubilised in Triton X-100 as described¹⁰ and PSU size measured¹¹ as the ratio of total chlorophyll to P700 (the reaction centre of photosystem I). The number of PSUs per cell was calculated from the cellular chlorophyll content and the PSU size¹¹. The spectral properties (300 and 77 K) of whole and detergent-solubilised membranes and fractions thereof were examined in an Aminco DW-2 spectrophotometer. Chlorophyll was determined in 80% acetone using the equations of Mackinney¹²; carotenoids were analysed using the methods of Davies¹³. Polyacrylamide gel electrophoresis of SDS-solubilised membranes was as described¹⁰ and distribution of chlorophyll in the pigmented gel zones was determined from gel scans¹¹.

To characterise the functional organisation of chlorophyll in the PSU of heterocysts and vegetative cells, spectral, photochemical and electrophoretic properties of Triton-solubilised membranes and the photosystem I reaction centre complex, the P700-chlorophyll *a*-protein, isolated therefrom were examined. In Triton-solubilised membranes of vegetative and heterocyst cells of both species, P700 photooxidation and dark reduction kinetics (see Fig. 2) were identical and indistinguishable from those described previously in other cyanobacteria and higher plants¹⁰. However, the wavelength of maximum absorbance decrease of P700 in Triton-solubilised membranes and the P700-chlorophyll *a*-protein occurred at 703 nm in contrast to 697 nm characteristic of P700 in higher plants and many cyanobacteria¹⁴. Previous investigations had demonstrated P700 in heterocysts by chemical difference spectra¹⁵ and by electron paramagnetic resonance spectroscopy¹⁶.

Thylakoid membranes from both cell types and species that were solubilised in SDS and electrophoresed in non-dissociating SDS polyacrylamide gels^{10,17} showed two pigmented zones. The slowest migrating zone was blue-green, stained for protein, and was identical in electrophoretic and spectral properties to the previously described SDS-altered form of the P700-chlorophyll *a*-protein or complex I. The second pigmented zone was green and did not stain for protein. It contained pigment, designated free pigment, that had been released from the native protein by detergent action. Complex I showed the same mobility in SDS gels and the same spectral properties whether isolated from

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Table 1 Distribution of chlorophyll, PSU size and relative numbers of PSU/cell in vegetative and heterocyst cells of *Nostoc* and *Anabaena*

	Heterocysts	Vegetative cells
<i>Nostoc muscorum</i>		
Chlorophyll (μg per cell)	0.7×10^{-7}	1.4×10^{-7}
PSU size (chlorophyll/P700)	60	180
PSU per cell (molecules P700 per cell)	4.0×10^5	2.6×10^5
<i>Anabaena cylindrica</i>		
Chlorophyll (μg per cell)	0.9×10^{-7}	1.7×10^{-7}
PSU size (chlorophyll/P700)	60	180
PSU per cell (molecules P700 per cell)	5.0×10^5	3.1×10^5

Values are the average of at least three experiments.

vegetative or heterocyst cells independent of species. In the vegetative cells, complex I accounted for 20–30% of the total chlorophyll of the thylakoids while in the heterocyst cells it accounted for 70–80%.

Triton-solubilised membranes were used for the isolation of the P700–chlorophyll *a*–protein by hydroxylapatite chromatography^{10,17}. The absorption and photochemical characteristics of this complex isolated from the two cell types and species (Fig. 2) revealed an absorption maximum at 677 nm and a maximum absorbance decrease due to P700 at 703 nm. Only the P700–chlorophyll *a*–protein from *Anabaena* heterocysts showed the lack of a characteristic 490-nm shoulder in its absorption spectrum (Fig. 2). This shoulder has been attributed to β -carotene^{10,19}; indeed, thin layer chromatography of this isolated complex confirmed the absence of carotenoids. The P700–chlorophyll *a*–protein typically contains about 40 ± 10 chlorophylls per P700 in most species thus far examined¹⁴, and pre-

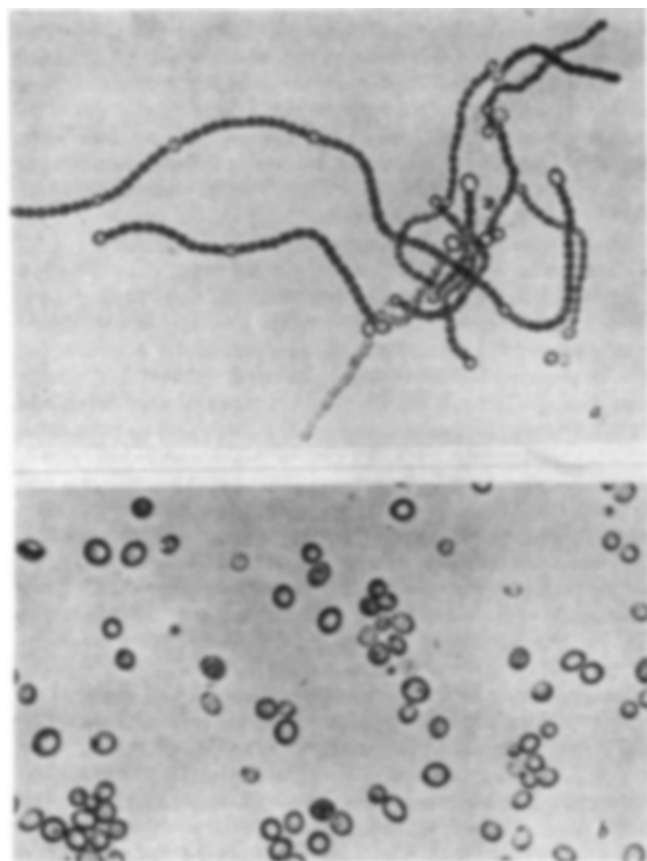


Fig. 1 *Nostoc muscorum*. a, Vegetative filaments showing terminal and intercalary heterocysts, the large, thick-walled cells. b, Isolated and purified heterocyst cells.

cisely that ratio in both cell types of *A. cylindrica* and *N. muscorum*. Thus, there seems to be no difference between the functional organisation of the centre of photosystem I in the different cells of the two cyanobacteria and that observed in other organisms.

Thylakoid membranes of both species solubilised in Triton X-100 revealed that vegetative cells have a similar chlorophyll to P700 ratio (180 ± 10) to that described previously for other cyanobacteria²⁰. Heterocyst thylakoids were more enriched in P700, exhibiting chlorophyll to P700 ratios of 60 ± 10 (Table 1). As heterocyst PSUs contain essentially no phycobilin pigments or phycobilisomes, the PSU size (ratio of total light-collecting tetrapyrrole chromophores to P700) in these cells is thus only about 60, compared to 300–350 (180 chlorophylls plus 120–180 phycobilin chromophores²⁰) in vegetative cells. Interestingly, the number of antenna molecules per reaction centre in heterocysts is the same small number found in many photosynthetic bacteria²¹. This large reduction in PSU size in heterocysts and the apparent enrichment of P700 is attributed both to the lack of

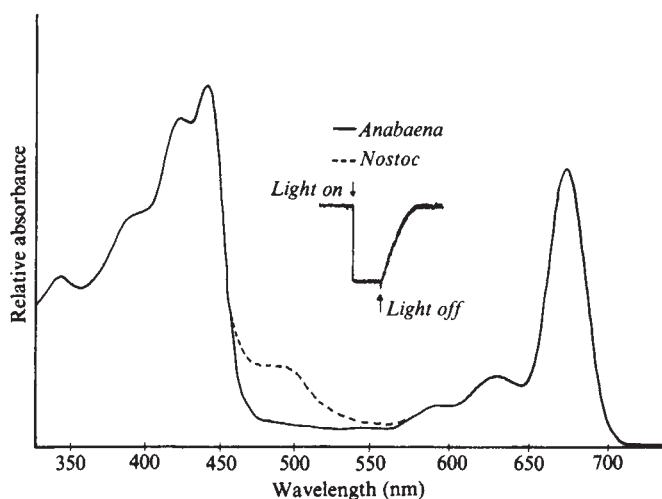


Fig. 2 Absorption spectra of the P700–chlorophyll *a*–proteins isolated from heterocyst thylakoids (at 20 °C) of *N. muscorum* (----) and *A. cylindrica* (—). Insert shows kinetics of photooxidation and dark reduction of P700 from *A. cylindrica* heterocyst P700–chlorophyll *a*–protein determined as described previously¹⁰.

phycobilin pigments, and to a two-thirds reduction in chlorophyll *a*; it closely correlates with the lack of photosystem II (refs 1, 2, 4). Thus much, if not all, of the chlorophyll and all of the phycobilins normally associated with photosystem II in vegetative cells²² is lost during heterocyst differentiation. The loss of detectable photosystem II activity may be related to the lack of phycobilisomes or phycobilin pigments and perhaps of photosystem II chlorophyll–proteins.

In comparison to vegetative cells, *Nostoc* and *Anabaena* heterocysts contain half the chlorophyll content, but have a PSU containing only a third of the number of chlorophyll molecules (Table 1). In both species the number of PSU per cell (Table 1) is 35% greater in heterocysts than in vegetative cells. Therefore, heterocysts show a considerable increase in the cellular content of photosystem I, an ATP and reducing power producing unit. Previous EPR studies¹⁶ also have suggested an enrichment in both P700 and photosystem I bound iron–sulphur proteins in heterocysts. The present study allowed for a direct measurement of the photosystem I content per cell. An increase in photosystem I units should lead to enhanced rates of cyclic photophosphorylation. However, photophosphorylation in heterocysts has been reported to be lower than that found in vegetative cells⁴. Because of the impermeability of isolated heterocysts to reactants, photophosphorylation can be measured only in ruptured cells and this probably contributes to

the lower than expected activity. Hence, until an *in vivo* assay can be developed, it will be difficult to assess photophosphorylation rates in heterocysts accurately.

Several conclusions can be drawn from the present study concerning the relationships between the functional organisation of the PSU and photosynthetic activities, and the N_2 -fixing role of heterocysts. It is clear that heterocysts are capable of supplying sufficient ATP and reducing power to drive N_2 fixation. Although these cells have fairly high rates of oxidative phosphorylation², it has been demonstrated^{4,23} that photosynthesis is the principal energy source of ATP for driving N_2 fixation. Heterocyst differentiation, therefore, should lead to the development of a thylakoid membrane suited for the energetic requirements of the cell. That this is the case follows from several lines of evidence. First, differentiation of heterocysts from vegetative cells results in pigment losses which seem largely restricted to photosystem II. Consequently, photosystem

II activity is eliminated as is production of O_2 , a potent inhibitor of nitrogenase. Second, the pigment alterations lead to the construction of a much smaller PSU; this probably allows for packaging of greater numbers of units within a given cell volume. Third, the reorganisation of the photosynthetic apparatus results in a greatly increased number of photosystem I units which can function to produce ATP and reducing power to support the energetic requirements for N_2 fixation. Finally, the lack of ribulose biphosphate carboxylase eliminates a major competitive system for ATP and reducing power. Hence, the modifications in the photosynthetic systems that occur during heterocyst differentiation seem to be specifically geared to the energetic requirements of these cells.

Research was supported by NSF grants PCM 78-15835 (to J.P.T.) and PCM 78-10535 (to R.S.A.), NSF-Energy related and NIH postdoctoral fellowships (to R.S.A.), a DOE grant (to L.P.), and an Andrew W. Mellon Fellowship (to R.S.A.).

Received 29 November 1979; accepted 13 February 1980.

- Haselkorn, R. A. *Rev. Pl. Physiol.* **29**, 319-344 (1978).
- Tel-Or, E. & Stewart, W. D. P. *Proc. R. Soc. B* **198**, 61-86 (1977).
- Bradley, S. & Carr, N. G. *J. gen. Microbiol.* **96**, 175-184 (1971).
- Tel-Or, E. & Stewart, W. D. P. *Nature* **258**, 715-716 (1975).
- Codd, G. A. & Stewart, W. D. P. *FEM Microbiol. Lett.* **2**, 247-249 (1977).
- Fay, P. *Arch. Mikrobiol.* **67**, 62-70 (1969).
- Clayton, R. K. *Light and Living Matter*, Vol. 2 (McGraw-Hill, New York, 1971).
- Tel-Or, E. & Stewart, W. D. P. *Biochim. biophys. Acta* **423**, 189-195 (1976).
- Allen, M. B. & Arnon, D. I. *Pl. Physiol.* **30**, 366-372 (1955).
- Shiozawa, J. A., Alberte, R. S. & Thornber, J. P. *Archs Biochem. Biophys.* **165**, 388-397 (1974).
- Alberte, R. S., McClure, P. A. & Thornber, J. P. *Pl. Physiol.* **58**, 341-344 (1976).

- Mackinney, G. *J. biol. Chem.* **140**, 315-322 (1941).
- Davies, B. H. in *Chemistry and Biochemistry of Plant Pigments*, Vol. 2 (ed. Goodwin, T. W.) 150-154 (Academic, New York, 1976).
- Thornber, J. P. A. *Rev. Pl. Physiol.* **26**, 127-158 (1975).
- Donze, M., Haveman, J. & Schiereck, P. *Biochim. biophys. Acta* **256**, 157-161 (1972).
- Cammack, R., Tel-Or, E. & Stewart, W. D. P. *FEBS Lett.* **70**, 241-244 (1977).
- Dietrich, W. E., Jr. & Thornber, J. P. *Biochim. biophys. Acta* **245**, 482-495 (1971).
- Cammack, R., Lujck, L. J., Maguire, J. J., Fry, J. U. & Packer, L. *Biochim. biophys. Acta* (in the press).
- Alberte, R. S. & Thornber, J. P. *FEBS Lett.* **91**, 126-130 (1978).
- Vierling, E. & Alberte, R. S. (in preparation).
- Thornber, J. P. *et al. Brookhaven Symp. Biol.* **28**, 132-148 (1976).
- Wang, R. T., Stevens, C. L. R. & Myers, J. *Photochem. Photobiol.* **25**, 103-110 (1977).
- Donze, M. *et al. Pl. Sci. Lett.* **3**, 35-41 (1974).

Grasses more sensitive to SO_2 pollution in conditions of low irradiance and short days

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Research into the effects of low levels of SO_2 on grasses has revealed no direct correlation between the dose of this pollutant and the plant's response¹. Authors have suggested that environmental factors are of prime importance in the control of the response, because in many cases damage was most severe during the winter^{2,3}. I have investigated the influence of total irradiance on the sensitivity of grasses to SO_2 , and shown that the effect of the pollutant was greatest in conditions of low irradiance and short photoperiod. Together, these factors may contribute to the increased sensitivity of plants during winter exposures to pollutants.

Experiments with a common pasture grass, *Phleum pratense* L. (Timothy), in a wind tunnel fumigation system had shown that plants grown at low irradiance, near the light compensation point, were more sensitive to 11 parts per hundred million (p.p.h.m.) SO_2 than plants grown under higher light levels (T.D., unpublished results). The experiment reported here was a continuation of that work, but irradiance levels and photoperiods representative of winter and summer conditions were used. Early in their growing season, grasses in Britain are exposed to days of about 12 h and low average irradiance, but during mid-summer, days of 16 h and higher levels of irradiance are experienced. Plants were therefore fumigated with SO_2 at these two day lengths, with a low irradiance of 40 W m^{-2} ($125 \mu\text{E m}^{-2} \text{ s}^{-1}$ photosynthetically active radiation, PAR), and higher irradiance of 130 W m^{-2} ($480 \mu\text{E m}^{-2} \text{ s}^{-1}$ PAR). Temperatures were the same for the two treatments, at 20°C (day) and 16°C (night), with a relative humidity of ~60%.

Seven-day old seedlings of Timothy variety S48 were fumigated in 'Perspex' chambers, volume approximately 0.08 m^3 , for

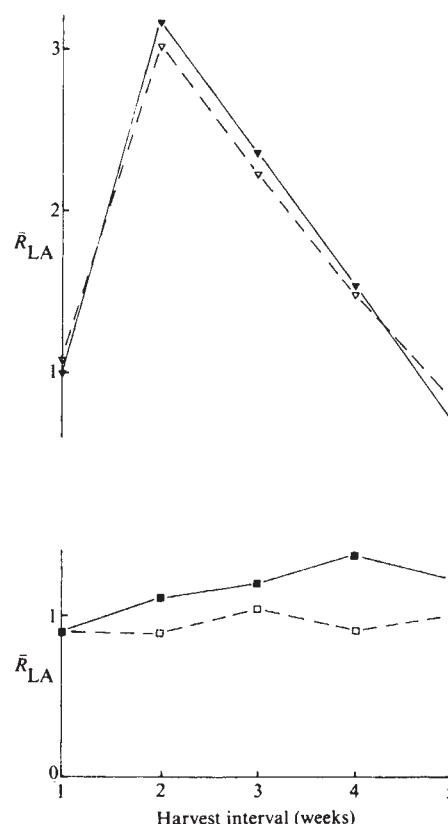


Fig. 1 Mean leaf area relative growth rates ($\bar{R}_{LA}$) for *Phleum pratense* L. with 12 p.p.h.m. SO_2 (----) or clean air (—). a, High irradiance, long day length; b, low irradiance, short day length.

5 weeks. Each chamber, containing 15 plants each in 7.5-cm pots, received air from a compressor at a rate of one complete air change per minute. In addition, a mixing fan situated inside the chamber rapidly circulated the air over the plants creating a turbulent wind speed of 0.4 m s^{-1} and good pollutant mixing.

Table 1 Final harvest data giving mean values for *Phleum pratense* L. grown under two light regimes with 12 p.p.h.m. SO₂ or clean air for 5 weeks

	No. of tillers	No. of green leaves	Green leaf area (mm ²)	Green leaf weight (g)	Dead leaf weight (g)	Stem weight (g)	Root weight (g)	Total shoot weight (g)	Total plant weight (g)
High irradiance, long day length									
SO ₂	8.12	26.99	12,291	0.3297	0.0006	0.1768	0.1966	0.507	0.704
Control	8.87	29.24	12,547	0.3384	0.0003	0.1653	0.2212	0.504	0.725
Reduction due to SO ₂	8%	8%	2%	3%	+143%	+7%	11%	+1%	3%
Probability	NS	NS	NS	NS	NS	NS	NS	NS	NS
Low irradiance, short day length									
SO ₂	1.08	3.23	274	0.0099	0.0008	0.0027	0.0015	0.013	0.015
Control	1.54	5.38	744	0.0199	0.0002	0.0060	0.0036	0.026	0.030
Reduction due to SO ₂	30%	40%	63%	50%	+355%	55%	58%	50%	50%
Probability	NS	0.1%	1.0%	0.1%	1.0%	0.1%	1.0%	0.1%	0.1%

NS, not significant.

Two chambers received 12 p.p.h.m. (343 µg m⁻³) SO₂ continuously, and two received clean air containing 0–1 p.p.h.m. SO₂; these acted as the controls. A pollutant concentration of 12 p.p.h.m. is representative of several British urban areas in winter, and does not exceed the highest monthly mean concentrations recorded in Britain⁴.

Each week the plants were removed temporarily and leaf length and width were measured, so that mean relative growth rates of leaf area, $\bar{R}_{LA}$, throughout the experiment could be calculated (Fig. 1). There was a large difference in relative growth rates between the two light regimes, but the pollutant had no effect in high irradiance and long days. With low irradiance, however, there was a significant reduction ($P < 0.5$) of $\bar{R}_{LA}$ between weeks 1 and 2, and weeks 3 and 4. This early interference in leaf growth could also be demonstrated by recording leaf lengths for the main shoot under low irradiance (Fig. 2). Little difference in leaf growth between SO₂ and control treatments was observed until after the second week of fumigation, when the first and second leaves had almost fully expanded. During the following weeks the senescence of these first two leaves was accelerated greatly by the presence of SO₂. The pollution also reduced the rate of extension of the third, fourth and fifth leaves, and reduced the mean maximum length of the third leaf. At harvest 5 there was no sign of the sixth leaf in the plants treated with SO₂, although in many of the control plants this leaf was quite well developed.

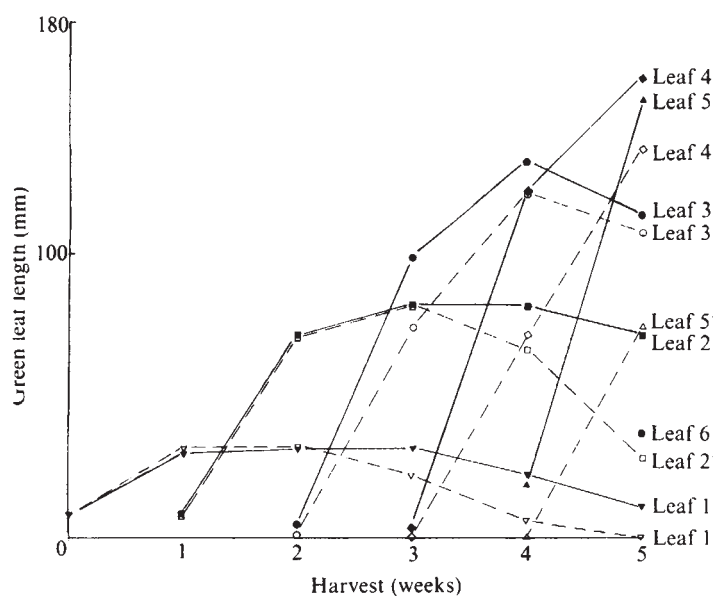


Fig. 2 Changes in green leaf length of *Phleum pratense* L. with time under low irradiance, short day length, when exposed to 12 p.p.h.m. SO₂ (-----) or clean air (—).

After 5 weeks' fumigation, results of the final harvest clearly demonstrated an effect of SO₂ on plant growth in conditions of low irradiance and short days (Table 1). All shoot and root parameters show ~50% reduction in dry matter accumulation in SO₂, and the green leaf area was particularly affected, for leaf senescence was also promoted. In contrast, SO₂ had no significant effect on the rapidly growing plants grown in conditions of high irradiance and long days. Plants growing in these conditions seem to be particularly resistant to SO₂.

The environmental factors of irradiance and/or day length have therefore had a large effect on the sensitivity of Timothy to 12 p.p.h.m. SO₂. Consideration of the biochemical activity of SO₂ within the cell may help to explain this effect. SO₂ readily dissolves on moist cell surfaces to form sulphite ions, SO₃²⁻, and the dissociation products HSO₃⁻ and H₂SO₃, depending on pH. Excess SO₃²⁻ is thought to be toxic to the cell in several ways: it may increase acidity and cause breakdown of chlorophyll *a*, promoting senescence⁵; electron flow within the chloroplasts may be disrupted⁶; SO₃²⁻ may competitively inhibit ribulose biphosphate carboxylase activity with HCO₃⁻, reducing photosynthetic capacity⁷; it has also been suggested that SO₃²⁻ forms addition compounds with sensitive proteins⁸, and that the imbalance caused between partially oxidised and reduced S compounds may inhibit cell division⁹. However, when SO₃²⁻ enters the cell, a large proportion may be oxidised to less toxic SO₄²⁻. This is the usual form in which sulphur is supplied to the plant through the roots, and which is readily assimilated in the chloroplasts. The SO₄²⁻ is metabolised by the sulphate reduction pathway to produce amino acids such as cysteine, methionine and glutathione. This process requires ATP, reduced ferredoxin and amino acid skeletons, all of which would be plentiful in a plant growing in conditions of high illumination and adequate nutrition. However, plants whose growth is severely limited by light will have low reducing power and will not be able rapidly to detoxify SO₂ products along this pathway. Toxic levels of SO₄²⁻ and SO₃²⁻ are, therefore, likely to accumulate and growth may be inhibited.

In the field, plants rarely grow in ideal unstressed conditions. In the experiment reported here, irradiance and/or daylength restrictions have been shown to increase greatly the sensitivity to SO₂ pollution, and other factors of environmental stress are important. Plants, therefore, seem to be more sensitive to SO₂ in the low-growth conditions of the winter, which is also when pollutant concentrations are greatest. Because laboratory studies tend to be conducted in conditions favourable to growth, the impact of atmospheric pollution on vegetation could be much greater than many results have indicated.

I thank NERC for financial support, and Professor T. A. Mansfield for supervision.

Received 17 December 1979; accepted 6 February 1980.

1. Bell, J. N. B., Rutter, A. J. & Relton, J. *New Phytol.* **83**, 627–643 (1979).
2. Bell, J. N. B. & Clough, W. S. *Nature* **241**, 47–48 (1973).
3. Ashenden, T. W. & Mansfield, T. A. *Nature* **273**, 142–143 (1978).

4. Warren Spring Laboratory, Ministry of Technology *The Investigation of Air Pollution. Monthly Summary* (1973).
5. Ricks, G. R. & Williams, R. J. H. *Envir. Pollut.* **8**, 97–106 (1975).
6. Nieboer, E., Richardson, D. H. S., Puckett, K. J. & Tomassini, F. D. in *Effects of Air Pollutants on Plants* (ed. Mansfield, T. A.) 61–85 (Cambridge University Press, London, 1976).
7. Ziegler, I. *Residue Revs* **56**, 79–105 (1975).
8. Garsed, S. G. & Read, D. J. *New Phytol.* **79**, 583–592 (1977).
9. Bleasdale, J. K. A. *Envir. Pollut.* **5**, 275–285 (1973).

In vivo* conversion of a labelled host plant chemical to pheromones of the bark beetle *Ips paraconfusus

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Chemical constituents of host plants have been suggested to affect in part the ability of certain insects to produce sex pheromones, and thus their ability to reproduce¹. We have investigated such a relationship between the bark beetle *Ips paraconfusus* (Coleoptera, Scolytidae) and its host tree *Pinus ponderosa*. The pheromone system of the insect is well characterised^{2–4} and, as we report here, myrcene, a constituent of the host oleoresin system, is converted in the male beetle to its pheromones, ipsenol and ipsdienol. We used deuterium labelling techniques² to demonstrate for the first time the unequivocal conversion of a host plant chemical to an insect pheromone.

I. paraconfusus males feeding on host phloem tissue produce three terpene alcohols which together are attractive to both sexes—ipsenol (Fig. 1, III), ipsdienol (Fig. 1, II) and *cis*-verbenol. Myrcene (Fig. 1, I) was used because previous studies^{5,6} had indicated that ipsenol and ipsdienol were produced in male but not female beetles when exposed to this terpene.

Myrcene (I) was labelled with deuterium by an unambiguous synthesis at the positions shown to avoid loss of deuterium on conversion to ipsenol or ipsdienol. The oxidation products could be identified by gas chromatography–mass spectroscopy (GC-MS). Myrcene-D was prepared by the method of Westmijze *et al.*⁷. Reaction of 5-chloropent-2-one with methyl-magnesium iodide D₃ produced 5-chlor-2-methyl-pent-2-ol. The alcohol was dehydrated with powdered sodium bisulphite to give 4-methyl-pent-3-enyl chloride (*cis* and *trans* isomers). The latter compound was converted to its cuprate and then added to vinylacetylene to produce myrcene-D. The identity of the products at each step was confirmed by NMR and IR and mass spectral analyses. The NMR of myrcene-D (Perkin Elmer R32, 90 MHz) indicated a deuterium content of approximately 1.6D in the methyl groups at 1.45 and 1.37 p.p.m. No deuterium was observable in any other position. Gas chromatography in conjunction with electron impact and chemical ionisation mass spectroscopy (EI- and CI-GC-MS) confirmed the presence of an isomeric mixture of myrcene-D₃, -D₂, and -D₁ with myrcene-D₂ as the predominant isomer. The loss of deuterium was explained by exchange during the dehydration step.

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Ten bark beetles of each sex were sealed in separate 20-ml ampules containing 10 µl of myrcene-D for 18 h as before⁶. Abdomens were then removed and crushed immediately in diethyl ether. Extracts were analysed by electron impact and chemical ionisation GC-MS on 10% Carbowax 20-m column using a Finnigan 4023 system. Peaks corresponding in retention time to ipsenol and ipsdienol, and possessing EI- and CI-GC-MS spectra consistent with the expected fragmentation patterns of ipsenol-D and ipsdienol-D, were observed in extracts of males, but not females. Further analysis by EI- and CI-GC-MS, using a SP-1000 30-m capillary column, confirmed the presence of the two deuterated compounds in male extracts which had coincident retention times with standard ipsenol and ipsdienol. With the exception of the diagnostic pattern of deuterium incorporation, the CI mass spectra of both compounds gave pseudo molecular ions (M+1) and fragmentation patterns similar to ipsenol and ipsdienol. The relative isotopic abundance of deuterium at M+1 for both compounds was also similar to that of deuterated myrcene. Clearly, these compounds were the hydroxylated products of labelled myrcene, deuterated ipsenol and ipsdienol.

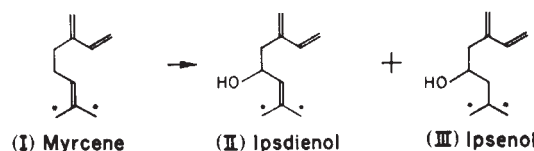


Fig. 1 Structures of myrcene, ipsdienol and ipsenol. Asterisks indicate positions of deuteration.

We conclude that male *I. paraconfusus* can convert myrcene, a terpene hydrocarbon from its host plant, to its pheromones, ipsenol and ipsdienol. Moreover, the presence of other labelled metabolic products in both sexes and the absence of labelled ipsenol and ipsdienol in the female demonstrate the sexual specificity^{2,6} of the biosynthesis of the pheromones. Although we cannot rule out completely alternative pathways (such as *de novo* pheromone biosynthesis, the conversion of other terpene hydrocarbons to pheromones, or induction of pheromone secretion), our results coupled with the observation by Hughes^{5,8} and Byers *et al.*⁶ that ipsenol and ipsdienol are found only in *I. paraconfusus* males exposed to myrcene, support the primacy of host chemicals in pheromone production. If Renwick's demonstration of enantiomeric specificity⁹ in the production of *cis*-verbenol (the non-sex specific component of the pheromone of male *I. paraconfusus*) on exposure to (–)- α -pinene is also a direct biosynthetic step, then all three known pheromone components may be derived from the host plant by simple oxidation of available chemicals. Experiments with isotopically labelled (–)- α -pinene may provide support for this premise. Thus, the evolution of host plant preferences in bark beetles may be a consequence in part of the chemistry of the tree as it affects pheromone biosynthesis.

This work was supported in part by grants from the Rockefeller Foundation (to L.B.H. and D.L.W.), and US Forest Service and Regional Research Project W-100, SEA/USDA (to D.L.W.). We thank Ron Skinner of Finnigan Corp. for technical expertise.

Received 24 September 1979; accepted 3 January 1980.

1. Hendry, L. B. *et al. Biochemical Interaction Between Plants and Insects*, 351–384 (Plenum, New York, 1976).
2. Fish, R. H., Browne, L. E., Wood, D. L. & Hendry, L. B. *Tetrahedron Lett.* **17**, 1464–1468 (1979).
3. Silverstein, R. M., Rodin, J. O. & Wood, D. L. *Science* **154**, 509–10 (1966).
4. Silverstein, R. M., Rodin, J. O., Wood, D. L. & Browne, L. E. *Tetrahedron* **22**, 1929–1936 (1966).
5. Hughes, P. R. *J. Insect Physiol.* **20**, 1271–1275 (1974).
6. Byers, J. *et al. J. Insect Physiol.* **25**, 477–482 (1979).
7. Westmijze, H., Kleijn, H., Meijer, J. & Vermeer, P. *Tetrahedron Lett.* 869 (1977).
8. Hughes, P. R. & Renwick, J. A. A. *Physiol. Ent.* **2**, 117–123 (1977).
9. Renwick, J. A. A., Hughes, P. R. & Krull, I. S. *Science* **191**, 199–201 (1976).

Association of lysophosphatidylcholine with fatty acids in aqueous phase to form bilayers

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Lysophospholipids have been implicated in a variety of physiological processes¹. *In vivo* lysophospholipids are invariably produced with fatty acids as the product of hydrolysis by phospholipase A₂ (ref. 2). Therefore, it is of considerable interest to examine the properties of the mixture of these lipids in aqueous suspensions. Results presented here demonstrate that a mixture of fatty acid and lysophosphatidylcholine forms a bilayer type of organisation even though the individual components form micelles when dispersed in an aqueous phase.

1-Acyl-lysophosphatidylcholines were prepared by the action of phospholipase A₂ on phosphatidylcholines in moist diethyl ether. Dispersions of appropriate lipid mixtures were prepared by a standard procedure that is used for the preparation of phospholipid liposomes³ and described in Fig. 1. Freeze-fracture electron microscopy was carried out according to well established techniques⁴. Glycerol was added to the samples to prevent freeze damage.

The aqueous dispersions of lysophosphatidylcholines with various fatty acids are cloudy. When inspected under a polarising microscope they exhibit birefringence, and a flow birefringence can be seen even at less than 10 μ M. Neither of these components dispersed in water alone exhibits such a behaviour^{5,6}. The most distinctive behaviour of mixtures of lysophosphatidylcholine and fatty acid in an aqueous phase is seen in their thermotropic transition profiles. As shown in Fig. 1 the equimolar mixtures of palmitoyl lysophosphatidylcholine with the various fatty acids exhibit a twin endothermic transition at characteristic temperatures that depend on the nature of the fatty acid. Thus, for homologous fatty acids the transition temperature and the enthalpy of transition increase monotonically with the chain length. Other data (not presented here) demonstrate that the transition temperature is lower for the mixtures containing fatty acids with double bonds, such as elaidic and oleic acids. Also, incorporation of cholesterol in equimolar ratios to lysophosphatidylcholine and fatty acids abolishes the transition completely, and substitution of the fatty acid by a long chain alcohol such as *n*-hexadecanol exhibits a twin transition at 327 K (7.45 Kcal mol⁻¹). Such observations demonstrate that the phase transition properties of these mixtures are predominantly governed by acyl chain interactions.

The birefringence and thermotropic phase transition behaviour of lysophosphatidylcholine and fatty acid mixtures suggests the existence of a bilayer-like phase that undergoes a cooperative phase transition. This is confirmed by ³¹P-NMR and freeze-fracture electron microscopy. The ³¹P-NMR spectra over a wide temperature range (one of these spectra is presented in Fig. 2) exhibit asymmetric peaks typical of a bilayer type of lipid organisation⁷. The effective chemical shift anisotropy (CSA_{eff}) is reduced compared with that for phosphatidylcholines. This is not uncommon for lysophosphatidylcholine present in bilayers of other lipids (unpublished observations). Furthermore, the CSA_{eff} is apparently temperature dependent, becoming less at higher temperatures, although a bilayer type of organisation is retained over the 277–327 K range we have examined. As shown in Fig. 3, the freeze-fracture faces obtained on the

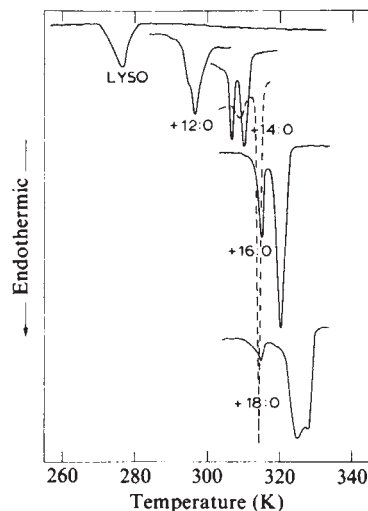


Fig. 1 The DSC scans of equimolar mixtures of palmitoyl lysophosphatidylcholine (LPC) with the fatty acids indicated as label: 12:O-, lauric; 14:O-, myristic; 16:O-, palmitic; 18:O-, stearic. The scans of palmitoyl lysophosphatidylcholine and dipalmitoyl phosphatidylcholine (broken line) are given for comparison. The experimental details are presented in the text. The transition temperatures and enthalpy for the various mixtures are: LPC (278 K, 3.6 kcal mol⁻¹); DPPC (314.6 K, 8.7 kcal mol⁻¹); LPC+12:O- (297 K, 3.3 kcal mol⁻¹); LPC+14:O- (307 K, 5.0 kcal mol⁻¹); LPC+16:O- (315.3 K, 5.7 kcal mol⁻¹); LPC+18:O- (324 K, 6.7 kcal mol⁻¹); LPC+elaidic acid (302 K, 4.7 kcal mol⁻¹); LPC+oleic acid (290 K, 4.5 kcal mol⁻¹). Appropriate amounts of lipids mixed in chloroform solution were dried under vacuum and then dispersed by vortexing in an aqueous buffer containing 50 mM KCl and 40 mM Tris at pH 8.0 in 25% aqueous ethylene glycol (v/v). Differential scanning calorimetric profiles were determined on 15- μ l samples containing 3 μ mol lipid mixture in sealed aluminium pans on a Perkin-Elmer DSC-2 instrument. The scan range for all runs was 240–340 K, and the scan rate was typically 5 K min⁻¹. Lower scan rates, repeated heating and cooling scans, and dispersions containing no ethylene glycol gave essentially identical results.

dispersions support the existence of vesicular structures of 1,000–6,000 Å diameter. Their fracture planes suggest the presence of bilayer orientation similar to that present in diacylphosphatidylcholine dispersions.

The phase properties of palmitoyl lysophosphatidylcholine and fatty acid dispersions in water closely resemble the phase properties of diacylphosphatidylcholines containing similar chains. Such a similarity could result if the acyl chains of lysophosphatidylcholine and fatty acid molecules associate to

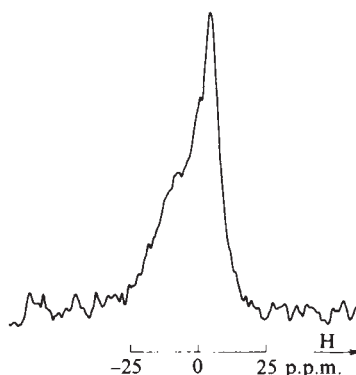


Fig. 2 The 36.4-MHz, high-power proton noise-decoupled ³¹P-NMR spectrum of an equimolar mixture of palmitoyl lysophosphatidylcholine and palmitic acid in 150 mM KCl, 10 mM Tris and 0.2 mM EDTA at pH 7.5 and 314 K. The spectrum was recorded using a spectral width of 12 kHz, 45° radio frequency pulses with pulse rate of 0.17 s and an exponential multiplication resulting in 50 Hz line broadening.

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form a complex that resembles diacylphosphatidylcholine, that is, in bilayers phosphatidylcholine may be considered as a conformationally and orientationally restricted analogue of fatty acid and lysophosphatidylcholine⁹. Although such a concept does not necessarily imply formation of a strong association complex, it is useful in explaining the behaviour of mixed lipid systems^{8,9} as well as the properties of bilayers modified by various solutes³.

A distinct phase behaviour of lysophosphatidylcholine and the fatty acid mixture is also observed in bilayers containing diacylphosphatidylcholine. Studies on aqueous dispersions of the ternary mixture containing equimolar lysophosphatidylcholine and fatty acid with varying mole fractions of diacylphosphatidylcholine (data not presented here) exhibit phase separation over a wide concentration range. Once again the behaviour of the mixture is quite distinct from that of diacylphosphatidylcholine containing either lysophosphatidylcholine or fatty acid⁶.



Fig. 3 Electron micrograph of the freeze-fracture replica of dispersions of palmitoylphosphatidylcholine with palmitic acid (1:1) $\times 50,000$.

The full implications of a distinct phase behaviour of fatty acid and lysophosphatidylcholine mixture are under investigation. However, the observations reported here do provide for the first time a physical basis for several yet unexplained observations: certain cell types and organelles have considerable amounts of lytic lysophospholipids but remain viable¹⁰. This could be due to the presence of equimolar proportions of fatty acids as demonstrated for adrenal chromaffin granules (R. Franson *et al.*, in preparation). The latency period during the action of phospholipase A₂ on bilayers is abolished in the presence of both the products of hydrolysis but not by either one of the products alone (unpublished observation). A distinctive behaviour of such ternary mixtures is probably best manifested by the bilayers and biomembranes treated with phospholipase A₂. For example, almost all the phospholipid in the outer monolayer of a bilayer can be hydrolysed with phospholipase A₂ without disrupting the vesicular structure¹¹; the red cells treated with phospholipase A₂ lyse only when they are treated with bovine serum albumin¹², which presumably extracts fatty acids from the membrane, and ³¹P-NMR spectra of phospholipase A₂-treated erythrocyte membranes exhibit retention of bilayer organisation¹³. Such observations suggest that lysophosphatidylcholine and fatty acids together retard haemolysis. Indeed, in the presence of fatty acids the time for lysophosphatidylcholine-

induced lysis is prolonged several hundredfold, and unsaturated fatty acids are more effective than the saturated fatty acids.

We thank Mrs J. Leunissen-Bÿvelt for the electron microscopy work.

Received 31 December 1979; accepted 8 February 1980.

1. Weltzien, H. U. *Biochim. biophys. Acta* **559**, 259–287 (1979).
2. Verger, R. & de Haas, G. H. A. *Rev. Biophys. Bioengng* **5**, 77–117 (1976).
3. Jain, M. K. & Wu, N. M. *J. Membrane Biol.* **34**, 157–201 (1977).
4. Ververgaert, P. H. J., Elbers, P. F., Luitingh, A. J. & van den Bergh, H. J. *Cytobiology* **6**, 86–96 (1972).
5. Klopfenstein, W. E., de Kruijff, B., Verkleij, A. J., Demel, R. A. & van Deenen, L. L. M. *Chem. Phys. Lipids* **13**, 215–222 (1974).
6. van Echteld, C. J. A., de Kruijff, B. & de Gier, J. *Biochim. biophys. Acta* **595**, 71–81 (1980).
7. Cullis, P. R. & de Kruijff, B. *Biochim. biophys. Acta* **507**, 207–218 (1978).
8. Jain, M. K. & White, H. B. *Adv. Lipid Res.* **15**, 1–60 (1977).
9. Jain, M. K., Ramirez, F., McCaffrey, T. M., Ioannou, P. V. & Marecek, J. F. *Biochim. biophys. Acta* (submitted).
10. de Oliveira Filgueiras, O. M., van den Besselaar, A. M. H. P. & van den Bosch, H. *Biochim. biophys. Acta* **58**, 73–84 (1979).
11. Wilschut, J. C., Regts, J. & Scherphof, G. *FEBS Lett.* **98**, 181–186 (1979).
12. Zwaal, R. F. A., Roelofs, B., Comfurius, P. & van Deenen, L. L. M. *Biochim. biophys. Acta* **406**, 83–96 (1975).
13. van Meer, G., de Kruijff, B., Op den Kamp, J. A. F. & van Deenen, L. L. M. *Biochim. biophys. Acta* **596**, 1–9 (1980).

Counting integral numbers of amino acid residues per polypeptide chain

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Proteins have integral numbers of each of the 20 amino acids. However, all the currently accepted methods of determining this number measure the ratio of moles of amino acid residue per mole of protein. This value is rarely close to an integer, due to experimental errors in determination of the molar amounts of both amino acid residues and polypeptide chain^{1–3}. A simple method which gives integral values of amino acid residues per polypeptide chain, independent of any other properties of the protein, would be useful in characterising proteins. We describe here one method; it is illustrated for the case of Cys residues, although the approach should be useful for many of the other 19 usual amino acids.

The method relies on the charge differences introduced by specific chemical modification of the amino acid. In Cys residues this is readily accomplished by reaction of the thiol group with iodoacetic acid, which introduces acidic carboxymethyl groups. Because of the chemical reactivity of thiol groups, Cys residues not reacted with iodoacetate are blocked with iodoacetamide, a similar, but uncharged, reagent.

A complete spectrum of protein molecules having 0, 1, 2, ..., n acidic carboxymethyl groups, where n is the integral number of Cys residues per protein molecule, is generated by reacting all the thiol groups with varying ratios of iodoacetamide to iodoacetate, using competition between the neutral and acidic reagents. To make all thiol groups of the protein chemically equivalent, the reaction is carried out in denaturing conditions, in this case, in 8 M urea.

The molecules with varying numbers of acidic groups are separated by an appropriate method; here by electrophoresis in conditions where the electrophoretic mobility is determined by the net charge of the protein. The number of species ($n + 1$), and hence the number of Cys residues (n), is determined by counting the number of bands.

The procedure is shown in Fig. 1a with bovine pancreatic trypsin inhibitor (BPTI), a small protein known to have six Cys

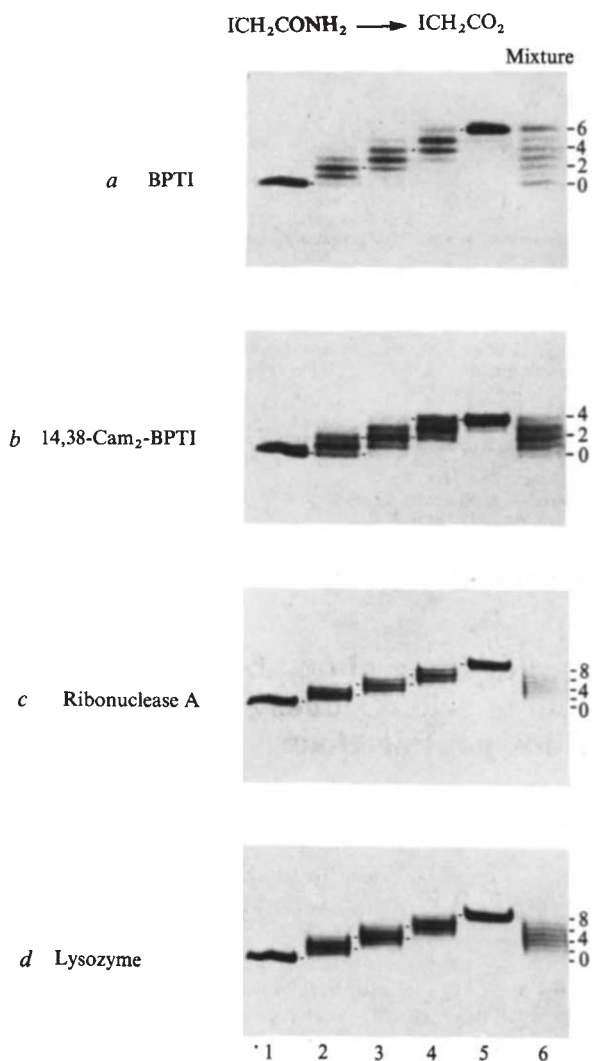


Fig. 1 Electrophoretic separation of protein molecules with 0 to n acidic carboxymethyl groups, where n is the number of cysteine residues in the protein. The reduced proteins were reacted with the neutral iodoacetamide (channel 1, on left), acidic iodoacetate (channel 5), and 1:1, 1:3, and 1:9 ratios of neutral to acidic reagents in channels 2, 3 and 4, respectively. Channel 6 (right) contains a mixture of equal portions of the samples applied to channels 1–5 in *a*, and of channels 2–4 in *b*, *c* and *d*. The bands are counted on the right by the number of acidic groups, and even-numbered bands are connected by dashes between them. The proteins (0.2 mg) were reduced and unfolded by incubation at 37 °C for 30 min in 1.0 ml 8 M urea, 10 mM dithiothreitol, 10 mM Tris-HCl, 1 mM EDTA, at pH 8.0. They were then alkylated by adding 200 μ l of this solution to 50 μ l of 0.25 M iodoacetamide, iodoacetate (adjusted to pH 8.0 with KOH), or mixtures of the two; after 15 min at room temperature, the solutions were placed on ice. Portions of 50 μ l were placed on polyacrylamide gels containing 8 M urea, using the low-pH discontinuous system of Reisfield *et al.*¹⁷; electrophoretic separation was at constant current (100–160 V) at 3 °C for 3–4 h. Staining was with 0.1% (w/v) Coomassie brilliant blue in 10% (w/v) trichloroacetic acid and 10% (w/v) sulphosalicylic acid overnight. The gels were destained by diffusion into 7.5% acetic acid and 5% methanol.

residues^{4–6}. The reduced protein in which the six thiol groups were blocked with iodoacetate migrates electrophoretically more slowly than that blocked with iodoacetamide, due to the six acidic carboxymethyl groups⁷. Reaction of the six thiols with mixtures of the neutral and acidic reagents in three different ratios generated the seven bands expected with six cysteine residues. Approximately equal competition between the two reagents was observed with a ratio of iodoacetate to iodoacetamide of 3:1, indicating that the latter reacts three

times as readily as the former, as has also been observed by others⁸. This mixture produced primarily molecules with 2, 3 and 4 carboxymethyl groups, so the entire spectrum was generated with threefold lower and higher ratios of reagents to generate significant amounts of molecules with 0, 1, 5 and 6 acidic groups. Molecules with 0 and 6 acidic groups were observed when the protein was reacted with just iodoacetamide and iodoacetate, respectively. All seven bands could be demonstrated simultaneously by making a mixture of the above populations.

BPTI, in which two Cys residues (residues 14 and 38) had been blocked covalently by iodoacetamide before competitive modification as described above, so that only four Cys residues were present^{9–11}, gave the expected five bands (Fig. 1*b*). Reduced bovine pancreatic ribonuclease and hen egg lysozyme, each with eight Cys residues^{12,13}, gave the expected nine bands in both instances (Fig. 1*c*, *d*). Bovine α -lactalbumin and β -lactoglobulin clearly demonstrated the presence of eight and five Cys residues, respectively, using an electrophoretic system for acidic proteins. Proteins with no Cys residues, such as the penicillinase of *Staphylococcus aureus*¹⁴, gave only a single band in all instances, with the same mobility when treated with either iodoacetamide or iodoacetate.

The procedure developed here for Cys residues is simple, rapid and gives integral values of the number of cysteine residues per polypeptide chain, independent of any other information about the protein, including its molecular weight. The procedure should be useful in determining the number of Cys residues of other proteins. It requires only a nearly homogeneous preparation of protein (which is also necessary, although not sufficient, for the determination of a correct value using currently accepted procedures) and a method of separating molecules with different numbers of carboxymethyl groups. The separation of species could be enhanced by the use of competition between acidic (iodoacetate) and basic (for example, ethyleneimine)¹⁵ reagents. Other means of separating such species, such as isoelectric focusing or ion-exchange chromatography, may be more appropriate with other proteins.

The general approach should also be useful for counting other amino acid residues for which there are specific reagents to introduce differences in net charge or other properties that may be used to resolve the species. Only a single reagent need be used, and the spectrum of molecules with 0 to n residues may be generated by varying either the time of reaction¹⁶ or the concentration of the reagent. Where two or more different reagents are available, competition between them may be used, as with iodoacetamide and iodoacetate here. The reaction should be carried out in conditions where the protein is unfolded and where all residues of the protein react at similar rates with the reagent. That this was the case with the Cys residues of the proteins studied here¹⁶ is demonstrated by the close agreement of the distributions of molecules with different numbers of carboxymethyl groups with distributions expected to random reaction of each of the Cys residues with the neutral and acidic reagents. The species generated must also be separated in unfolding conditions, where the modified residues have an equivalent effect on the separation procedure. For example, the band of BPTI with one carboxymethyl group per molecule contains molecules in which that acidic group is on any of the six Cys residues of the protein¹⁶, so all six isomeric species must have the same electrophoretic mobility. The other bands must also contain large numbers of species with the carboxymethyl groups distributed over the six Cys residues. Yet the bands are as sharp as those of the homogeneous species with 0 and 6 carboxymethyl groups and are equally spaced.

Any such modification procedure will depend on the selectivity of the reagent and on obtaining stoichiometric reaction of the residues of the pertinent amino acid in limiting conditions, which should be apparent by comparison of the mixtures at varying extents of reaction. Stoichiometric reaction should be indicated by approaching formation of a single species after extensive reaction, as is the case in Fig. 1. Slower reaction at

other sites should be apparent by the slower generation of additional, multiple bands after very extensive reaction.

The procedure developed here will not replace the standard methods of amino acid analysis, as it is unlikely that specific methods will be available for specifically modifying those amino acids with no reactive groups, for example glycine, proline, valine, leucine. Nevertheless, it should be useful for calibrating the molar ratios determined by amino acid analysis, using the integral values determined for one or a few convenient amino acids.

Bayer provided purified BPTI (Trasylol). We thank Miss Denise Thomas for assistance and R. H. Pain for supplying *Staphylococcus aureus* penicillinase.

Received 12 December 1979; accepted 13 February 1980.

1. Light, A. & Smith, E. L. in *The Proteins* Vol. 1, 2nd edn (ed. Neurath, H.) 1–44 (Academic, New York, 1963).
2. Schroeder, W. A. *The Primary Structure of Proteins* (Harper and Row, New York, 1968).
3. Konigsberg, W. H. & Steinman, H. M. in *The Proteins* Vol III, 3rd edn (eds Neurath, H. & Hill, R. L.) 1–178 (Academic, New York, 1977).
4. Kassell, B. & Laskowski, M. Sr *Biochem. biophys. Res. Commun.* **20**, 463–468 (1965).
5. Anderer, R. A. & Horne, S. J. *biol. Chem.* **241**, 1568–1572 (1966).
6. Huber, R., Kukla, D., Ruhlmann, A., Epp, O. & Formanek, H. *Naturwissenschaften* **57**, 389–392 (1970).
7. Creighton, T. E. *J. molec. Biol.* **87**, 579–602 (1974).
8. Webb, J. L. *Enzyme and Metabolic Inhibitors* Vol. III (Academic, New York, 1966).
9. Kress, L. F. & Laskowski, M., Sr. *J. biol. Chem.* **242**, 4925–4929 (1967).
10. Meloun, B., Fric, I. & Sorm, F. *Colln Czech. chem. Commun.* **33**, 2299–2306 (1968).
11. Liu, W. & Meienhofer, J. *Biochem. biophys. Res. Commun.* **31**, 467–473 (1968).
12. Smyth, D. G., Stein, W. H. & Moore, S. J. *biol. Chem.* **238**, 227–234 (1963).
13. Canfield, R. & Liu, A. K. J. *biol. Chem.* **240**, 1997–2002 (1965).
14. Ambler, R. P. *Biochem. J.* **151**, 197–218 (1975).
15. Raftery, M. A. & Cole, R. D. J. *biol. Chem.* **241**, 3457–3461 (1966).
16. Creighton, T. E. *J. molec. Biol.* **96**, 777–782 (1975).
17. Reisfield, R. A., Lewis, U. J. & Williams, D. E. *Nature* **195**, 281–283 (1962).

Brillouin scattering, density and elastic properties of the lens and cornea of the eye

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Brillouin spectra of biological systems may ultimately be related to their intrinsic molecular properties. In some instances the optical properties may be associated with the elastic ones and ultimately with the force constants of the molecules involved^{1,2}. In the present work we have used a triple-pass Fabry–Perot interferometer to measure Brillouin light scattering spectra for refractive tissues of the eye, including cornea, capsule and lens. Combined with corresponding measurements of density, estimates of the real and imaginary parts M' and M'' of the longitudinal bulk modulus have been made for the first time. Measurements have extended over four classes of vertebrate: Mammalia, Aves, Pisces and Amphibia; only small differences have been found between the various samples of cornea, whereas marked differences occur between the different lenses. Hence this account concentrates largely on the latter. The implications of this work lie not so much at the ophthalmological level as at the macromolecular and offer, in conjunction with other scattering techniques, opportunities for probing the lens and its proteins topographically as a function of growth.

The development, morphology, biochemistry, optics and vision of normal vertebrate eyes have been studied for many years^{3–7}, but comparatively few investigations of the elastic

properties of lenses have been made and none by the method presented here^{8–14}.

Brillouin scattering^{15,16} arises when light is scattered by the periodic fluctuations in density that occur in condensed matter as a result of thermally excited hyperfrequency sound waves. The scattered light is shifted in frequency from that of the incident light by typically 1–10 GHz for 90° scattering because of the interaction of the incident light waves with the coherent longitudinal sound waves. The simplest Brillouin spectrum of an isotropic substance therefore consists of the central unshifted Rayleigh line together with two satellites of lower and higher frequency respectively. Aqueous gels of low macromolecular content (~1% by weight) will give Brillouin shifts differing only slightly from that of water, whereas gels of high protein content lead to substantial shifts (ref. 17 and our unpublished data). The high protein content of corneal and lens tissues makes them particularly suitable for the present measurements.

From the differential equation governing the propagation of a longitudinal acoustic wave in a viscoelastic medium the complex longitudinal modulus M^* is given by¹⁸

$$M^* = M' + iM'' = \rho u^2 + i(2\rho u^3 \alpha / \omega) \quad (1)$$

whence

$$M' = \rho u^2 \quad (2)$$

and

$$M'' = 2\rho u^3 \alpha / \omega \quad (3)$$

where ω is the angular frequency of the shift, u the speed of hypersound in the medium α the attenuation defined by the relation

$$\Delta = \alpha u / \pi \quad (4)$$

where Δ is the corrected width of the Brillouin line (FWHM in GHz).

In the arrangement used in these experiments^{2,19} the Brillouin shift Ω is related to the hypersonic speed and the wavelength of incident radiation λ_0 by

$$\Omega = \pm 2n_1 \frac{u}{\lambda_0} \sin \beta \quad (5)$$

when n_1 is the refractive index of air and 2β the scattering angle in air ($90^\circ \pm 1^\circ$).

Provided the density of the sample is known one can therefore determine the longitudinal compressive moduli M' and M'' from measurements of Brillouin scattering data. Characteristic results are summarised in Fig. 1A and B and Table 1.

Collection of data on the lens of the normal human eye has been limited by scarcity of suitable material. The spectra shown in Fig. 1A were taken from one of the two globes so far made available to us and at a time before the development of the density-measuring technique (see legend to Table 1).

The Brillouin shift observed for corneal material, ~4.5 GHz, is lower than that for rat-tail collagen^{1,2}. This is almost certainly due to the fact that the cornea is a complex tissue of collagen, proteoglycans and water. All biological specimens so far examined (including bovine serum albumin, data not shown) show marked increases in Brillouin shift on drying.

Before discussing the data on lenses summarised in Table 1 it is important to note the mode of development through the differentiation of their epithelial cells. Elongated cells are produced at the cortex; and as new layers are formed older ones move inwards. In man and primates this leads to a lens structure of concentric layers of closely apposed fibre cells. In general, the protein content, density and refractive index of the lens increases along any radial line joining periphery to centre. The lens is in broad terms a complex protein gel, the overall protein content of which is about one-third of the lens mass. The crystallins, which are globular in character and comprise four predominant classes α , β , γ and δ . Each class contains a number of separable

proteins. Of these the γ class is a closely similar group of cryoprotein monomers of low molecular weight ($\sim 20,000$), while the α and β crystallins (with the exception of β_2) are heteropolymers. The distribution, proportions and molecular weights of the crystallins in the lens change during development as new fibres of different composition are laid down.

Turning to Table 1 and Fig. 1, it is clear that in all lenses studied so far values of density, hypersound speed and elastic modulus (as here defined) all increase from the lens periphery to the centre of the nucleus. This is consistent with Bettelheim and Wang's²⁰ corresponding observations of topographic variation of refractive index in the lens of the calf. In man, cow and sheep there are comparatively small changes of lens density and Brillouin shift between periphery and nucleus. This contrasts sharply with the case of the rat for which a nuclear Brillouin shift of ~ 8.8 GHz has been observed. The quasi-spherical lens of the rat is also substantially different in shape from the generally oval character of those found in the other mammals investigated. The lens of the fowl is rather soft and there is not much difference in elastic modulus between periphery and nucleus. With the bony fishes we come again to lenses almost spherical in shape and with very hard nuclei with appropriately high values of M' . The data for *Sarotherodon mossambicus* (Fig. 1B) and *Perca fluviatilis*

(Table 1) are typical in showing high Brillouin shifts for lens nuclei which are always physically more obvious than in the mammals (other than rodents) and the fowl.

It is interesting to compare the present values of M' of about $3 \times 10^9 \text{ N m}^{-2}$ with the values of Young's modulus for the human lens measured by Fisher^{8,9}. For essentially static distortions values of $3 \times 10^3 \text{ N m}^{-2}$ for the lens and 10^6 N m^{-2} for its isolated capsule were found. We should emphasise that our own values relate to a microscopic scale and that the light scattered is effectively probing high frequency sound wave propagation at wavelengths $< 1 \mu\text{m}$ which is smaller than recorded measurements of lens fibre width. Note that in his lens measurements Fisher takes an average value for lens density, whereas in our work values for small volumes of tissue are measured at positions corresponding to the recorded Brillouin shifts. Our values of longitudinal bulk modulus (M') are of similar magnitude to those found recently for collagen^{1,2}. Kikkawa and Sato¹¹ and Ejiri *et al.*¹² have not given values of the modulus and their results can thus not be compared with the present work.

Fisher and Pettet²⁵ have reported that the lens nucleus in man contains $63.4\% \pm 2.9\%$ water by weight and the cortex $68.8 \pm 4.3\%$. In some animals (Table 1) the nuclear density approaches that of crystalline proteins²⁶ where values of water content by

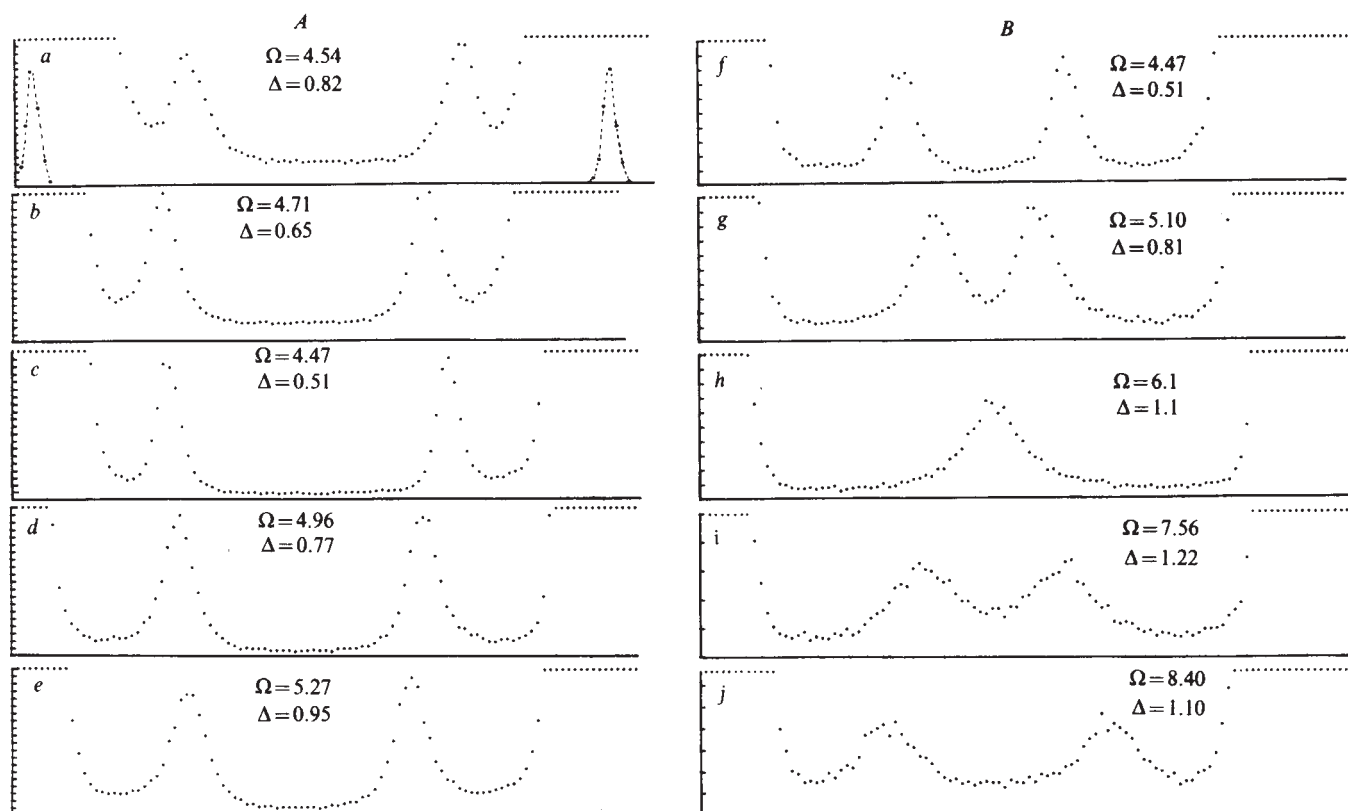


Fig. 1 Eyes were removed from animals within an hour of death and kept in liquid paraffin over ice until the material could be isolated for experiment. In these conditions the cornea and lens tissues showed little spectral change over a period of ~ 2 days. After removal from the globe, material was mounted between microscope slides separated by near-airtight spacers of different thicknesses, but usually $\sim 250 \mu\text{m}$. Samples of cornea were generally thicker than this and with a little Ringer solution gave a good optical seal to the slides and consequently good transmission of the incident laser beam. Two types of lens preparation were employed: either small excised portions $\sim 1 \text{ mm}^3$; or, where spherical as in fish and rat, the whole tissue. The slides were mounted as described previously^{2,19}. With this arrangement the refractive index is not required and the measurements are made at a constant scattering vector $\mathbf{K}$ where $|\mathbf{K}| = (4\pi n_1 \sin \beta)/\lambda_0$ (here equal to $1.82 \times 10^7 \text{ m}^{-1}$). **A**, Brillouin spectra of eye material from man and sheep. The recordings show one complete order with free spectral range 17.0 GHz; instrumental peaks from elastic scattering are superimposed and shown dotted at the top (and greatly reduced). The Brillouin peaks are frequency shifted from their nearest elastic peak: *a*, human cornea (and instrumental peaks); *b*, periphery, human lens; *c*, capsule, sheep lens; *d*, periphery, sheep lens; *e*, nucleus, sheep lens. The lens of the human eye which was examined ~ 4 d post mortem showed a strong yellow fluorescence characteristic of age of subject and its rather cloudy appearance gave very strong elastic scattering. The full scale ordinate is 2,000 counts per channel and the channel width is 173 MHz; the measured shifts and reduced line widths (FWHM) in GHz are shown. **B**, Brillouin spectra recorded at different radial depths in one specimen of the spherical lens of the teleost fish *Sarotherodon mossambicus*. The lens diameter is $\sim 2.0 \text{ mm}$ and the free spectral range 12.2 GHz. The Brillouin shift increases sharply through the series from periphery to nucleus and the peaks in *i* and *j* relate to the further elastic peak. The depths are *f*, 0.06 mm; *g*, 0.28 mm; *h*, 0.52 mm; *i*, 0.76 mm; *j*, 1.0 mm (at lens centre). The measured shifts and reduced line widths (FWHM) in GHz are shown; the intervals on the ordinate scale are 100 counts per channel and the channel width is 122 MHz.

Table 1 Examples of Brillouin scattering, density and elastic moduli for eye tissues of Mammalia, Aves, Pisces and Amphibia

Animal	Tissue	Density ρ (kg m^{-3})	Brillouin shift $\Omega(\text{GJz})$	Spectral width Δ	Hypersound velocity (from equation (2)) u (km s^{-1})	Absorption coefficient $\pi\Delta/u$ ($\times 10^6 \text{ m}^{-1}$)	Longitudinal	
							elastic M' ($=\rho u^2$) ($\times 10^9 \text{ N m}^{-2}$)	modulus M'' ($=2\rho u^3\alpha/\omega$) ($\times 10^8 \text{ Nm}^{-2}$)
Cow (<i>Bos</i> sp.) (1 yr 9 months)	Cornea	1.107	4.43	0.87	1.52	1.8	2.56	5.1
	Lens: periphery	1.105	4.93	0.83	1.70	1.5	3.20	5.3
	Lens: nucleus	1.248	5.36	1.02	1.85	1.7	4.27	8.0
Rat (<i>Rattus</i> sp.) (19 weeks)	Lens: periphery	1.144	5.60	1.09	1.93	1.8	4.26	8.4
	Lens: nucleus	1.358	8.78	1.02	3.03	1.1	12.5	15
Bird								
Domestic fowl <i>Gallus</i> sp. (30 weeks)	Lens: periphery	1.082	4.61	0.51	1.59	1.0	2.73	3.0
	Lens: nucleus	1.124	4.91	0.61	1.69	1.1	3.21	3.9
Fish (teleost): perch <i>Perca fluviatilis</i> (~7 months)	Lens: periphery	1.103	4.93	0.59	1.70	1.1	3.19	3.8
	Lens: nucleus	1.238	8.75	1.09	3.02	1.1	11.3	14
Amphibian: common frog <i>Rana temporaria</i> (mature)	Lens: periphery	1.108	4.50	0.45	1.55	0.9	2.66	2.6
	Lens: nucleus	1.118	6.6	1.00	2.28	1.4	5.81	9.0

The passively stabilised triple pass interferometer²¹ used in these experiments was essential to bring out the weak Brillouin scattering features observed in the presence of strong elastic scattering at the incident frequency. The light source was an argon ion laser operating in a single mode at 488 nm and at power levels varying between 5 and 20 mw. For most of the material studied the Brillouin scattering intensity was comparable with that of pure water, and spectra with about a thousand counts per channel in each peak could be accumulated in about 10 min. Within the sample, the laser beam was focused to ~30 μm diameter and scattered light was collected from ~25 μm length along the beam, corresponding to a volume of $\sim 1.4 \times 10^{-4} \text{ mm}^3$. The corneas were found to become slightly cloudy within 12 h post mortem and very strong elastic scattering ~10⁶ times the Brillouin scattering intensity was evident. Material from lens and capsule generally showed 1–2 orders less elastic scattering. A gradient density column (originally introduced by Linderström-Lang and Lanz²² as modified by Low and Richards²³ has been used to measure density using a mixture of xylene ($\rho = 865 \text{ kg m}^{-3}$) and chloroform ($\rho = 1,498.4 \text{ kg m}^{-3}$) in a small closed measuring cylinder coated internally with 'Repelcote' to prevent adherence of tissue. The preparation of the column and precautions required followed the lines of the earlier work and will not be elaborated here. Accurate measurements of density can readily be obtained in this way, but it is more difficult to determine precisely the position within the lens from which tissue has been removed and techniques are being developed to overcome this. The values of density given for nuclei are for the nucleus as a whole in rat, fish and frog; in other mammals and birds the specimen was excised from the centre. Attempts to fragment the denser nuclei by cutting slices frequently resulted in complete opacity and it cannot be assumed that the density in this state is normal. The calibration droplets of KBr solutions were checked with a Paar densimeter²⁴.

volume of ~43% occur most frequently and may range from 27 to 65%. In old rat lenses protein concentrations in the nucleus may reach values as high as 0.9 g ml^{-1} corresponding to a water content of only 35% by volume²⁷. The high values of Brillouin shift in certain lens nuclei thus seem to correlate well with high protein concentration and low water content.

Note that in weak gels at low concentrations the Brillouin scattering technique would measure hypersound wave propagation in the solvent, corresponding to ~1,400 m s^{-1} or ~4.3 GHz. For low concentrations ($c \approx 1\%$) the essential propagation in the gel network is slow; speeds up to 10 m s^{-1} have been found by Brenner *et al.*²⁸ in agarose gels, with elastic moduli varying as $c^{4.1}$ (their equation (4)). A large extrapolation to concentrations of ~35% suggests a speed in the gel lattice of the order of that found for the solvent. This establishes that at even higher protein concentrations as in the lens nucleus the propagation must be essentially dominated by the gel and its structure.

In conclusion we have used Brillouin scattering to determine for the first time the longitudinal high frequency elastic modulus of a substantial variety of vertebrate lenses. This in combination with other scattering techniques summarised below offers an

additional approach to the study of the biophysics of lenses on a molecular scale.

The use of a range of incident light-wave frequencies and scattering vectors as well as variation of temperature would allow the investigation of dispersive effects in lens material and further clarify the differences between the static results of Fisher⁸ and our own dynamic studies. There is also some promise that the use of Raman spectra²⁹ may add precision to the knowledge of protein content and secondary structure in lenses as a function of position and development. X-ray diffraction studies of lens nuclei in particular may help to understand the properties of this high-density material. The use of individual crystallins of the vertebrate eye as model systems is now possible and makes the investigation of the effect of variation of water content on Brillouin spectra of lens proteins an important prospect.

We thank Karen M. Mundell for the measurements of density; R. M. Clayton and C. J. Randall for helpful discussions; and Professor M. S. Laverack, D. B. Marshall Ltd, D. H. Mills, Professor C. I. Phillips and Professor R. J. Roberts for material. J.T.R. is in receipt of a grant from the MRC.

Received 6 September 1979; accepted 8 February 1980.

- Harley, R., James, D., Miller, A. & White, J. W. *Nature* **267**, 285–287 (1977).
- Randall, J. T. & Vaughan, J. M. *Phil. Trans. R. Soc.* **293**, 133–140 (1979).
- Walls, G. L. *The Vertebrate Eye* 1–785 (Cranbrook Institute of Science, 1942).
- Davson, H. (ed.) *The Eye* 2nd edn, Vols 1–6 (Academic, New York, 1969–1976).
- Clayton, R. M. *Curr. Topics dev. Biol.* **5**, 115–180 (1970).
- Bloemendal, H. *Science* **197**, 127–138 (1977).
- Pumphrey, R. J. in *The Cell and The Organism* (eds Ramsay, J. A. & Wigglesworth, V. B.) 193–208 (Cambridge University Press, 1961).
- Fisher, R. F. *J. Physiol., Lond.* **212**, 147–180 (1971).
- Fisher, R. F. *J. Physiol., Lond.* **201**, 1–19 (1969).
- Fisher, R. F. *J. Physiol., Lond.* **270**, 51–74 (1977).
- Kikkawa, Y. & Sato, T. *Expl Eye Res.* **2**, 210–215 (1963).
- Ejiri, M., Thompson, H. E. & O'Neill, W. D. *Vision Res.* **9**, 233–244 (1961).
- Fincham, E. *Br. J. Ophthalm. Suppl.* **VIII** (1937).
- Weale, R. A. *Br. J. Ophthalm.* **46**, 660–668 (1962).

- Brillouin, L. *C. r. heb. séanc. Acad. Sci., Paris* **158**, 1331–1334 (1914).
- Brillouin, L. *Ann. Phys.* **17**, 88–122 (1922).
- Bedborough, D. S. & Jackson, D. A. *Polymer* **17**, 573–576 (1976).
- Litovitz, T. A. & Davis, C. M. in *Physical Acoustics: Principles and Methods* Vol. IIA, Pt. A. (ed. Mason, W. M.) 281–290 (Academic, New York, 1965).
- Vaughan, J. M. *Phys. Lett.* **58A**, 325–328 (1976).
- Bettelheim, R. A. & Wang, T. J. Y. *Expl Eye Res.* **18**, 351–356 (1972).
- Food, R., Pomeroy, W. R. M. & Vaughan, J. M. *RSRE Res. Rev.*, Paper 8 (1977).
- Linderström-Lang, K. & Lanz, H. R. *Lab. Carlsberg, Ser. Chim.* **21**, 316–324 (1938).
- Low, B. W. & Richards, F. M. *J. Am. chem. Soc.* **79**, 1660–1666 (1952).
- Kratky, O., Leopold, H. & Stalringer, H. *Meth. Enzym.* **27**, 98–110 (1973).
- Fisher, R. F. & Pettet, B. *J. Physiol., Lond.* **234**, 443–447 (1973).
- Matthews, B. W. *J. molec. Biol.* **33**, 491–497 (1968).
- Phillips, B. *Invest. Ophthalm.* **8**, 258–270 (1969).
- Brenner, S. L., Gelman, R. A. & Nossal, R. *Macromolecules* **11**, 202–207 (1978).
- Yu, N.-T., East, E. J. & Chang, R. C. C. *Expl Eye Res.* **24**, 321–334 (1977).

MATTERS ARISING

A new tectonic model for the central Norwegian Caledonides

HORNE'S¹ recent model for the evolution of the central Norwegian Caledonides requires that the Storen, Hovin and Horg Groups originated as an intra-oceanic arc complex above a westerly dipping subduction zone. It is difficult to reconcile present knowledge of the volcanic and sedimentary rocks of these groups with this hypothesis. The Storen Group metavolcanics are well established as being ocean floor basalts² obducted onto the Baltic Shield in pre-Arenig times³. In the Løkken-Meldal region a younger complex of basic volcanics of late-Arenig age occurs within the Lower Hovin Group⁴. These are considered to have originated in a back-arc marginal basin situation⁵. Calc-alkali volcanics of similar age occur some 100 km further west³. The Lower Hovin Group locally contains meta-andesites, but these are volumetrically less important than the rhyolitic volcanics which predominate in the upper Lower Hovin, Upper Hovin and Horg Groups^{4,6,7} (mid-Ordovician to early Silurian). In the Løkken-Meldal region the volcanoclastic sandstones of the Lower Hovin Group have a quartz content that is considerably higher⁴ than that considered typical of an island arc environment⁸. The sandstones and shales of the Upper Hovin Group have a chemistry and mineralogy consistent with a rhyolitic/granitic (continental?) provenance (unpublished data). The Horg Group contains quartzites and vein quartz pebble conglomerates^{6,7}. Thus neither the volcanic nor the sedimentary rocks of the Storen, Hovin and Horg Groups are typical of those expected in an ensimatic arc complex. Indeed the presence of considerable rhyolitic volcanic products is

often considered to imply an Andean type margin^{9,10}. Also the distribution of late-Arenig vulcanism suggests an arc to the west with a marginal basin opening to the east—this requires subduction of the opposite polarity to that proposed by Horne¹.

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1. Horne, G. S. *Nature* **281**, 267–270 (1979).
2. Gale, G. H. & Roberts, D. *Earth planet. Sci. Lett.* **22**, 380–390 (1974).
3. Furnes, M., Roberts, D., Sturt, B. A., Thon, A. & Gale, G. H. *Proc. int. Ophiolite Symp., Geol. Surv. Cyprus* (in the press).
4. Ryan, P. D., Skevington, D. & Williams, D. M. *Proc. IGCP Symp.*, Blacksburg (US Geological Survey (in the press)).
5. Gale, G. H. & Pearce, J. A. *Can. J. Earth Sci.* (in the press).
6. Vogt, T. H. *Norsk. geol. Tidsskr.* **25**, 449–527 (1945).
7. Chaloupsky, J. N.G.U. **266**, 277–304 (1969).
8. Crook, K. A. W. *Soc. Econ. Palaeont. Miner. Spec. Publ.* **19**, 304–311 (1974).
9. Presnall, D. C. & Bateman, P. C. *Bull. geol. Soc. Am.* **84**, 3181–3203 (1973).
10. Myashiro, A. *Am. J. Sci.* **274**, 321–355 (1974).

HORNE REPLIES—Ryan has raised some important and difficult problems of provenance and petrogenesis that persist in other parts of the Appalachian-Caledonian orogen as well as in the Trondheim region. I look forward to evaluating the unpublished papers he cites as evidence, particularly those pertaining to the age of obduction and the polarity of subduction.

Many of Ryan's arguments are based on the composition of extrusive and detrital rocks in the Løkken-Meldal area some 60 km west of Selbusjøen. Although I have not studied those rocks I am reluctant to accept generalisations based on petrotectonic pigeonholing when it is well established that volcanics in modern arcs and even some spreading ridges are so variable in composition^{1,2}. The compositions of the volcanic and sedimentary strata that Ryan describes from the Løkken-Meldal area are similar to those of strata of comparable age exposed in the

Bronson Hill anticlinorium of New England^{3,4}. Rhyolites are common in the Ordovician volcanic succession, and they are thought to have been the original core rocks of the Oliverian mantled gneiss domes⁵. Many of the Ordovician and Silurian psammitic strata along the anticlinorium are quartzose, and the early Silurian Clough quartzite that rests unconformably on the volcanics and gneiss domes is conglomeratic with very common pebbles of vein quartz within orthoquartzite matrix⁶. Although there is no easy solution to the provenance problem here or in the Trondheim region, most workers regard the rocks in the Bronson Hill anticlinorium as originating on an ensimatic arc complex within a closing Iapetus Ocean^{7,8}.

The calc-alkaline volcanics in western Norway possibly do represent an arc complex further west than the Trondheim arc. However, the presence of neither a western arc nor ophiolites in the Bergen area⁹ helps resolve the ambiguity of subduction polarity in the Trondheim region. Was there a western Iapetus seaway that closed into a second arc?

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1. Myashiro, A. *Am. J. Sci.* **274**, 321–355 (1974).
2. Walker, G. L. P. *Bull. volcan.* **29**, 375–406 (1966).
3. Billings, M. P. *Bull. geol. Soc. Am.* **48**, 463–566 (1937).
4. Rodgers, J. *The Tectonics of the Appalachians*, 91–115 (Wiley-Interscience, New York, 1970).
5. Naylor, R. S. in Zen, E., White, W. S., Hadley, J. B. & Thompson, J. B. *Studies of Appalachian Geology—Northern and Maritime*, 231–240 (Wiley-Interscience, New York, 1968).
6. Thompson, J. B., Robinson, P., Clifford, T. N. & Trask, N. J. in *Studies of Appalachian Geology—Northern and Maritime* (eds Zen, E., White, W. S., Hadley, J. B. & Thompson, J. B.) 203–218 (Wiley-Interscience, New York, 1968).
7. Bird, J. M. & Dewey, J. F. *Bull. geol. Soc. Am.* **81**, 1031–1060 (1970).
8. Williams, H. *Tectonic Lithofacies Map of the Appalachian Orogen*, (Memorial University, Newfoundland, 1978).
9. Sturt, B. A., Thon, A. & Furnes, H. *Geology* **7**, 316–320 (1979).

Corrigendum

In the letter '(ADP-ribose)_n participates in DNA excision repair' by B. W. Durkacz *et al.*, *Nature* **283**, 593–596, in Fig. 2 the symbols are incorrectly defined. It should read '△, 0 min; ▲, 40 min; ●, 80 min; and ○, 300 min'. Lines 16–17 of Fig. 2 legend should read 'a, b. The cells were exposed to DMS for 20 min, which was washed out, but 3 mM 3-aminobenzamide was added to the medium for the 300 min recovery period. c, d. The cells were exposed only to DMS'.

Errata

In the letter 'Plasmid-mediated tissue invasiveness in *Yersinia enterocolitica*' by D. L. Zink *et al.*, *Nature* **283**, 224–226, a line was omitted from paragraph 4. Line 4 should read '... Serotype 0:8 strains contained either a plasmid of molecular weight (MW) 35 × 10⁶, a plasmid of MW 41 × 10⁶ or both (whereas...)'.

In the letter 'Do genealogical patterns in purple photosynthetic bacteria reflect interspecific gene transfer?' by C. R.

Woese *et al.*, *Nature* **283**, 212–214, the x axis in the upper part of Fig. 1 should be labelled S_{AB} and the x axis in the lower part should be labelled % Sequence homology.

In the letter 'The relationship between coding sequences and function in haemoglobin', by W. A. Eaton, *Nature* **284**, 183–185, on p. 184 second paragraph line 11 should read '...(2) Residues in the α₁β₂ contact'.

BOOK REVIEWS

Impressive inhibitors

Michael Cannon

THIS book is published by Wiley-Interscience as a sequel to *Nucleoside Antibiotics* which appeared in 1970. It describes the application of naturally occurring nucleoside and nucleotide analogues as biological probes in cellular reactions. The compounds listed and considered inhibit between them an impressive range of events including cell wall synthesis, protein synthesis, DNA and RNA synthesis, purine and pyrimidine interconversions, and the activities of both cyclic-AMP-phosphodiesterase and adenosine deaminase. Several of the compounds are of medical importance since they have limited use in the treatment of cancer and viral infections in humans.

It is of no surprise to find an old friend — puromycin — featured in the text. Many biochemists have successfully experimented with this most useful antibiotic to study the mechanism of peptide bond formation on ribosomes, the release of incomplete nascent polypeptide chains and the properties of the ribosomal binding sites for transfer RNA. What may be surprising to some, however, is the fact that there are 69 other compounds described here of which 40 or so have been discovered since the 1970 volume was published. The number of additional events inhibited has increased dramatically in line with the number of new compounds identified. Medical implications are also clearly indicated. In 1970, for example, only 5-azacytidine, of the nucleoside antibiotics, had been used to treat cancer, showing good activity against certain leukaemias and limited activity on some solid tumours, although predictably the drug frequently produces unpleasant side effects. Of the newer compounds described in the present volume, both the pyrrolopyrimidine nucleoside antibiotic tubercidin and 9- β -D-arabinofuranosyladenine (ara-A), the latter having low myelosuppressive activity in humans, have been cleared by the Federal Drugs Administration for the treatment of human basal cell carcinoma, herpes

Nucleosides as Biological Probes. By R. J. Suhadolnik. Pp.364. (Wiley: New York and Chichester, UK, 1979.) \$50, £23.

simplex keratitis and herpes simplex encephalitis. Tubercidin is also a potent anthelmintic agent, as is the pyrimidine nucleoside disaccharide analogue hikizimycin (anthelmycin).

The presentation in this book is fairly standard for each entry and usually covers discovery, production, isolation, physical properties, chemical and biological properties, structural elucidation, biosynthesis, toxicity and where known the mode(s) of action. The 'interest generation' is extremely variable. Some compounds come over as fascinating and remarkable inhibitors. Thus coformycin and 2'-deoxycoformycin inhibit adenosine deaminase, an important enzyme in purine metabolism, a deficiency of which produces an inborn error of metabolism associated with severe combined immunodeficiency disease. The role of these two compounds as immunosuppressants aids the success of tumour grafts and indicates a usefulness in organ transplantation. Other compounds, by contrast, come over as being somewhat boring but it is fair to say that in each case beauty, or lack thereof, will always be in the eye of the beholder.

All but two of the ten chapters end with a particularly valuable summary section drawing together many of the important facts in concise form. Of the two chapters lacking a summary one deals exclusively, albeit briefly, with clitidine and tells us very little of the biological properties of this pyrimidine nucleoside, although we do discover that it resides in the mushroom *Clitocybe acromelalga* and is a physiologically active substance. One presumes that little is, in fact, known about this compound. The remaining summary-less chapter deals equally briefly with the herbicides A and B, 5'-O-glycosyl-ribonucleosides, raphanatin and 6-benzylamino-7- β -D-glucopyranosylpurine. Curiously we are told that the herbicides

show promise as weed killers but are not toxic to mice (or gardeners?).

The text is profusely illustrated not only with the chemical structures of the various compounds described but also with a large selection of diagrams and tables reproduced from original papers. I can understand the author's wish to underscore his factual writing with real experimental data but I found this aspect of the book very annoying on occasion. Certainly these entries fill out the volume but much of the original experimental data presented are, in my opinion, redundant. For example, I am prepared to accept the author's word that cordycepin (3'-deoxyadenosine) inhibits the synthesis of ribosomal RNA and if I found this observation of particular interest I should naturally read the relevant reference. I see little reason why the present text should labour the point by illustrating an experiment involving agarose gel electrophoresis of ribosomal RNA from L1210 cells treated with the inhibitor. Again, an illustration is hardly necessary to support the statement that whereas RNA polymerases isolated from either *Escherichia coli* or T3 bacteriophage are not affected by α -amanitin, the former enzyme is completely inhibited by the naturally occurring nucleotide analogue thuringiensin whereas the latter enzyme is not. There are many other such examples throughout the text.

Overall, however, this will be a useful volume for those who are interested in the subject area — although whether or not the number of such individuals represents a viable economic proposition for Wiley-Interscience, and hence for Professor Suhadolnik, is debatable. Certainly, though, the text makes the reader aware of the remarkable versatility of nucleoside and nucleotide analogues with respect to their inhibitory effects and allows for interesting speculation in the fascinating field of structure-function relationships.

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Mammalian vestibular physiology

R.H.S. Carpenter

Mammalian Vestibular Physiology. By V. J. Wilson and G. Melvill Jones. (Plenum: New York and London, 1979.) £20.48.

WHY do neurophysiologists choose to study one part of the brain rather than another? Amongst sensory systems, at least, it seems to be those which give us the greatest sensual enjoyment that attract the closest investigation: if we add to this the inevitable band-waggon effect, the result is a strikingly disproportionate amount of effort on vision and to some extent hearing, and rather little on anything else. The vestibular system has fared particularly badly from this: apart from the ambiguous delights of the roller-coaster and sky-diver, the system comes to most people's notice only at times of distinct misery — a bad Channel crossing, the acute effects of alcoholic overindulgence — and its contribution to the unending task of keeping us precariously balanced on two tiny supports goes largely unnoticed and unappreciated.

Of course, it has to be admitted that the vestibular system is somewhat redundant, in the sense that much of the information about head position and movement that it provides is also furnished (though more slowly) by the visual system and by proprioceptors in our joints. A man whose vestibular system has been destroyed by disease may not be aware that much is wrong until these alternative sources of information are also unusable — running over rough ground in the dark, or swimming underwater. But as Wilson and Melvill Jones make abundantly clear, these parallel visual and proprioceptive inputs seem largely to make use of the neural circuitry that was originally evolved to process vestibular information; and the study of central vestibular pathways has shed a good deal of light on how information from the eyes and other receptors is used for the same purpose.

One aspect of this cooperation that is particularly interesting, the importance of which has only recently been fully appreciated, is the way in which the matching of visual and vestibular signals is a function that must be *learned* by the brain: this calibration of one input in terms of the other seems to be going on all the time, ensuring that degenerative and other changes in the performance of the vestibular organs do not result in inappropriate responses. A dramatic way of demonstrating this is to get a subject to wear prisms that reverse his visual field: after only a matter of days it is

found that certain of his purely vestibular responses — ones that can be evoked in the dark — follow suit by reversing their direction as well. These changes are almost certainly the result of functional alterations in cerebellar circuitry, and offer the exciting possibility of studying motor learning by the cerebellum in a simple and quantifiable way.

Mammalian Vestibular Physiology provides a first-class account not only of these more recent areas of interest but also of the classical anatomy and physiology of the vestibular organs and their central connections, and fills much more than adequately what has long been an obvious gap in the literature. It is sufficiently wide

in its scope to be of interest to neurophysiologists in other areas as well as to specialists in vestibular and oculomotor physiology, who will certainly find both its intelligent and impartial reviews of difficult topics, and its notably comprehensive bibliography, of more than ephemeral value. Perhaps it may also help to lure neurophysiologists away from the rather obvious charms of visual physiology to a field which promises to begin to unveil the more challenging mysteries of the motor system. □

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Laser fusion

Siegbert Witkowski

The Physics of Laser Fusion. By H. Motz. Pp.290. (Academic: London and New York, 1979.) £17; \$37.

IT is a rather bold undertaking to write a textbook on the physics of laser fusion, for essentially two reasons. Firstly, laser fusion is a very rapidly developing field. The interpretation of the complex phenomena has only reached a quasi-steady state which has not yet been proven to be stable against new experimental results. Secondly, the physics of laser fusion comprises a large variety of quite different topics. Therefore, selection of the proper material for an introduction to this field is a major problem. The author has succeeded in this difficult task. He has chosen the essentials, concentrating on the basic phenomena, which are well established. The physics of the nuclear reactions and of high power laser systems

are only touched on briefly in the introductory chapters in order to acquaint the reader with the problems involved and the state of the art. The main part of the book deals with light plasma interaction processes, the excitation, propagation and interaction of waves in plasmas and their relevance to laser fusion. The complex phenomena connected with energy transport in the overdense region are touched on in a special chapter preceding the treatment of the hydrodynamics and its stability. Besides the analytical treatment of the phenomena, results from computer codes are also presented. Each chapter is followed by a list of references where more detailed information can be found on specific subjects. In conclusion, this book can be recommended as an introduction to the field of laser fusion for newcomers, but it is also a useful compilation of the basic physics of laser fusion for the specialist. □

Siegbert Witkowski is a member of the Board of Directors, Projektgruppe für Laserforschung, Munich, FRG.

Models of r and d

Alan Grafen

The Natural Selection of Populations and Communities. By D.S. Wilson. Pp.186. (Benjamin Cummings: Menlo Park, California, 1980.) \$12.95.

IN whose interest does an animal or plant behave? Evolutionary theorists believe that the workings of natural selection ensure that organisms act as if they were maximizing their reproduction (or more precisely, a weighted sum of their own and their relatives' reproduction). So although some kinds of behaviour might easily be explained as an individual acting in the interests of some larger unit such as its

group, species or community, the orthodox view is still that only in extreme and implausible conditions can this type of explanation be admitted. In *The Natural Selection of Populations and Communities*, D.S. Wilson attempts to give a theoretical justification for accepting these 'higher' levels of selection.

This book contains much to commend it to biologists interested in natural selection. Wilson's discussions, in the introduction and elsewhere, of the logic of his attempt are excellent. The idea of "trait groups" as the population subdivision within which frequency dependence operates,

contrasted with the "deme" within which there is free interbreeding, had seemed a contrivance, but the book convinced me of its biological interest. His concept of the "neutral pathway" is very stimulating. The examples are fascinating and show that the debate is about animals and not algebra, and the burying beetles in particular provide a challenge to ordinary, individual selection thinking.

A very valuable aspect of a book in which every main point is made with a model is that disagreements about it can be precisely stated. Wilson makes two controversial claims about his structured deme model which I believe to be mistaken. The first is that it "routinely" allows the evolution of a kind of altruism not predicted by the orthodox, individual selection model. The second is that species should act more in the interests of the community than the individual selection model predicts, because of "indirect effects". This second claim underlies the whole second half of the book. I believe that in making these important claims, Wilson has been misled by subtleties in his own algebraic formulations. The models supporting the two claims are very similar, and the following argument about "weak altruism" can be easily applied to "indirect effects".

To explain this rather important error, I will describe briefly the conclusions of the algebraic model. The effect of a trait is that its possessor has d more offspring while

every other member of the trait group has on average r more offspring. (Often either d or r will be negative.) Wilson first shows that in the individual selection model the trait will spread if $d > r$. This is because the trait group is the deme and a possessor of the trait must do better than average in the deme.

The condition for spread in the structured deme model depends on how the deme is divided into trait groups. If each group has exactly the same proportion of possessors of the trait — the identical groups case — then it is $d > r$. If possessors and nonpossessors of the trait are divided randomly into groups then it is $d > 0$. Wilson points out that there is a class of traits with $r > d > 0$ which will spread with random grouping but not with identical grouping, and these he calls "weakly altruistic". So far so good.

He goes on to identify the individual selection model with the identical grouping case of his structured deme model, and I believe this is an error. He concludes that the individual selection model is very special and implausible, and that we should expect to see animals behaving more generously towards each other than the individual selection model predicts. This is an important claim.

In both the individual selection model and the identical groups case the condition for spread is $d > r$, so how can Wilson be mistaken in identifying them? Because r must be interpreted differently in the two

cases. In the individual selection model every other member of the *deme* gains r but in the identical groups case only the other members of the *trait group* do. To see the significance of this difference, consider how the random groups case should be understood in terms of the individual selection model. The trait benefits each other member of the group by r , but it is the average effect on other members of the *deme* we need to use in the individual selection model. Wilson assumes that there are many groups in the deme, and so when the group members' r values are averaged over the whole deme the result will be very small. The condition for spread in the individual selection model then becomes nearly $d > 0$, even though r may be reasonably large. (In fact, the condition in the random groups case itself is only "nearly" $d > 0$.)

The random groups case, therefore, corresponds to the orthodox, individual selection model, and "weak altruism" is not, after all, a major contribution of structured deme theory to evolutionary thought. And, by a similar argument, neither is the concept of "indirect effects".

In conclusion, this book contains a very stimulating and clearly presented argument for using 'higher' levels of selection; but an argument which is seriously flawed. □

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Human internal clocks

J.T. Enright

The Circadian System of Man. By R.A. Wever. Pp.276. (Springer: Berlin, Heidelberg and New York, 1979.) DM98; \$53.90.

THIS monograph summarizes the results from some twelve years of experimental studies on human circadian rhythms, conducted in a unique underground facility at the laboratories of Professor Jürgen Aschoff in Bavaria. Data are available from more than 200 experiments, with an average duration of about a month each, during which the subjects — usually singly but sometimes in groups of 2 to 4 — lived in complete temporal isolation from the outside world. A few dozen comparable experiments have been conducted by others, elsewhere, but Wever's data base is such that this treatise can be taken as a definitive statement of present knowledge on the topic. Many of these results, which involve both free-running and entrained rhythms, have been previously published as a series of technical reports; nevertheless, there are conspicuous

advantages to having everything available in the present compact form: a well organized and systematic consideration of all the data, rather than interim, single-topic progress reports.

For me, the most interesting issue involved in these studies is the comparison of human circadian rhythms with those of other well studied vertebrates. Probably the most conspicuous difference is the extreme lability sometimes seen in the wake-sleep patterns of human subjects, superimposed on a more stable background of regular cycles in body temperature and other 'vegetative' rhythms. One hamster or mouse or finch seldom differs from the next by more than an hour in the free-running period of its wake-sleep rhythm, but some 20% of the human subjects had grossly non-circadian wake-sleep patterns (periods as short as 16 hours and as long as 60 hours). Subjects who thought they were living a normal routine occasionally undertook 'sleep deprivation' without even being aware of it: sustained wakefulness of up to 48 hours, while the temperature rhythm went on with its 24- to 25-hour oscillation. Another of the interesting differences between humans and other vertebrates, probably reflecting our domestication, is that lighting regimes — the overwhelmingly strongest synchron-

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izing agents for the circadian rhythms of other animals — apparently are only subsidiary in humans to the stronger effects of various sorts of social synchronization.

Wever himself has supervised all the massive programme of research described here, and the interpretations of the data are, of course, his own. Some of those interpretations are inevitably less convincing than others, and each reader will no doubt find certain issues about which he has reservations. It strikes me, for example, as over-interpretation, when the phase relationship between the body-temperature rhythm and the wake-sleep cycle is taken not just as a hint, but as proof that a subject's rhythm was accidentally synchronized by some unintended environmental cycle (p.38). I also wonder what to think about the implications of electromagnetic fields for the rhythms: small differences in mean period, correlated with shielding from natural electromagnetic fields, were statistically significant during the first 5 years of experimentation ($n = 84$); but I find it distressing

to see that the effect was not reproduced during the subsequent 8 years of studies (p.96; $n = 58$). An imposed 10-Hz square-wave electric field was associated with a shorter circadian period for 12 of 17 subjects tested (the other 5 excluded because of "internal desynchronization"); but in subsequent experiments, a 10-Hz sinusoidal field led to equivocal results (p.114). This difference can in principle be attributed to higher harmonics in the square-wave signal — or does it instead reflect on the reproducibility of the initial finding?

But these and similar questions are minor matters. Regardless of details in the interpretation, this monograph offers a wealth of data; it is an irreplaceable source book for those interested in the experimental study of human circadian clocks. □

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Igneous petrology fifty years on

R. N. Thompson

The Evolution of the Igneous Rocks. Fiftieth Anniversary Perspectives. Edited by H.S. Yoder Jr. Pp.588. (Princeton University Press: Guildford, UK, 1979.) Hardback £19.30; paperback £8.40.

IN 1928 N. L. Bowen wrote his masterpiece *The Evolution of the Igneous Rocks*. This book caused a revolution in the philosophy and approach of igneous petrology by demonstrating that many aspects of the behaviour of magmas could be explained adequately by straightforward application of chemical principles, without the addition of the pinch or two of geological magic dust so frequently used by other theoreticians of Bowen's time. The 18 eminent petrologists who have contributed to the fiftieth anniversary volume are all the inheritors of Bowen's approach to magma chemistry. Each was asked to take a chapter of the original book and describe what has happened in that part of the subject during the following half century. As might be expected, there are great differences in the ways in which the various authors have approached their task. Some of them alternate extensive quotations from Bowen, or paraphrases of his ideas, with balanced summaries of the present state of knowledge on each theme. In contrast, a few of the contributors have been unable to resist the temptation to use their chapters almost solely as vehicles for summarizing their own work and reiterating their own views; in one article

there is no mention at all of either Bowen or his book.

Taken as a whole, the many excellent articles far outweigh the few others. The chapters that I found particularly interesting were on silicate liquid immiscibility (Roedder), crystal accumulation and sorting (Irving), siliceous potassic glassy rocks (Stewart), volatile constituents (Burnham) and petrogenesis (Wyllie). There are also many sections of valuable data and discussions in other chapters and I anticipate that this book is one which almost every working igneous petrologist or geochemist will wish to buy. On the other hand, it is probably of only limited use to the wider readership of students. The reason for this lies in the terms of reference of the volume. Rigorous adherence by the contributors to the themes in igneous petrology which Bowen chose in 1928 has inevitably led to omission, or virtual omission, from the 1979 book of any subjects which were neglected or completely unrecognized 50 years ago. This policy leads to a curious overall impression in the book, because many aspects of current igneous petrology are discussed in detail, whilst other important themes, such as crystallization kinetics, and trace-element and isotope geochemistry, are almost completely omitted. The irony is that, if Bowen had rewritten his own book in 1979, these are exactly the sorts of forward-looking applications of chemical principles to igneous petrology that he would have emphasized. □

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Selective electrodes

J.A.W. Dalziel

Working with Ion-Selective Electrodes. By K. Cammann. Pp.225. (Springer: Berlin, Heidelberg and New York, 1979.) DM 76, \$41.80.

THIS monograph, a worthy successor to others that have appeared, is a translation from a second German edition. It reviews 447 publications up to 1977. The author, after experience in the applications department of an instrument manufacturer, claims an understanding of the problems that face the users of ion-selective electrodes. He considers that their difficulties arise mainly from lack of knowledge and remedies this in two ways — first, with a theoretical treatment to serve as reference in understanding the action of electrodes in potentiometry generally, and then by offering practical advice on their construction, mode of operation and a survey of their present range of applications.

Particular emphasis is given to consideration of pitfalls in the reproducible measurement of electrode potentials, with advice on reference electrodes, buffers and liquid junction potentials directed towards stable e.m.f. measurements of high impedance cells. The different categories of ion-selective electrodes (solid-state glass, homogeneous and heterogeneous membranes, supported ion exchanger and neutral carrier membranes, and electroactive coatings, gas-diffusion and bio sensors, but without the glass pH electrode which is intentionally omitted) are all described in turn with full practical details, including methods of construction.

The second half of the book has sections on techniques and applications. Besides calibration and the direct measurement of activity (concentration) under static conditions, much space is devoted to titration procedures with indicator electrodes, using the Gran extrapolation and other methods which allow for the slow response of some electrodes near the end point. Whether such methods would be chosen for routine situations is questionable. The short section on continuous measurements in industry and environmental research should be more interesting, but in fact the progress reported is rather disappointing when one remembers that it is over 15 years since 'new' ion-selective electrodes were made. For the future, the important step will be to discover better ways of interfacing the electrolytic species in sample solutions so that they react directly and selectively with electronic charge carriers within semiconducting electrodes. □

J.A.W. Dalziel is Reader in Analytical and Inorganic Chemistry at Chelsea College, University of London, UK.

BOOKS RECEIVED

Mathematics

- BARENBLATT, G. I. Similarity, Self-Similarity, and Intermediate. Asymptotics. Pp. xvii + 218. ISBN-0-306-10956-5 (New York and London: Consultants Bureau, 1979.) \$42.00
- GLADWELL, I. and WAIT, R. (ed. by). A Survey of Numerical Methods for Partial Differential Equations. Pp. x + 424. ISBN-0-19-853351-9. (Oxford: Clarendon Press, Oxford University Press, 1979.) £13.50
- GRAHAM, Alexander. Matrix Theory and Applications for Engineers and Mathematicians. Pp. 295. (Chichester: Ellis Horwood Limited, 1979.) £15.75.
- KNOPFMACHER, John. Analytic arithmetic of algebraic function fields. Pp. v + 130. ISBN-0-8247-6907-4. (New York and Basel: Marcel Dekker, Inc., 1979.)
- ROBERTS, F. S. Measurement Theory with Applications to Decision-making, Utility, and the Social Sciences. Encyclopaedia of Mathematics (Massachusetts, London, Amsterdam, Ontario, Sydney, Tokyo: Addison-Wesley, 1979.) \$24.50.

Astronomy

- BURTON, W. B. The Large Scale Characteristics of the Galaxy. Proceedings of IAU Symposium No. 84. Pp. xviii + 611. ISBN-90-277-1029-5 hardback ISBN-90-277-1030-9 paperback. (Dordrecht, Boston, London: D. Reidel, 1979.) Dfl. 140 US\$73.50 hardback; Dfl. 70 US\$36.85 paperback.
- LANG, Kenneth R. and GINGERICH, Owen (ed.). A Source Book in Astronomy and Astrophysics, 1900-1975. Pp. xx + 922. ISBN-0-674-82200-5. (Cambridge, Massachusetts and London: Harvard University Press, 1979.) \$50.00.
- KRUGER, A. Introduction to solar Radio Astronomy and Radio Physics. Pp. xv + 324. ISBN-90-277-0957-2 hardback; ISBN-90-277-0997-1 paperback (Dordrecht, Boston, London: D. Reidel, 1979.) Dfl. 95 US\$49.95 hardback Dfl. 45 US\$23.60 paperback.

Physics

- CHARNEY, Elliot. The Molecular Basis of Optical Activity. Pp. x + 364. ISBN-0-471-14900-4. (New York, Chichester, Brisbane, Toronto: John Wiley & Sons, 1979.) £16.80.
- GUSEV, N. G. and DMITRIYEV, P. P. Quantum Radiation of Radioactive Nuclides. A Data Handbook. Pp. v + 483. ISBN-0-08-023058-X. (Oxford, New York, Paris, Frankfurt: Pergamon, 1979.) US\$100 £50.00.
- PALMADESSO, P. J. and PAPADOPOULOS, K. (ed.) Wave instabilities in Space Plasmas, Proceedings of a Symposium organized within (Dordrecht, Boston, London: D. Reidel, 1979.) Dfl. 75 US\$39.50.
- PEIERLS, R. Surprises in Theoretical Physics. Pp. viii + 166. ISBN-0-691-08241-3 hardback ISBN-0-691-08242-1 paperback (Princeton, New Jersey: Princeton University Press, 1979.) £8.60 hardback £2.20 paperback.
- ZIMAN, J. M. Principles of the Theory of Solids. Pp. xviii + 435. ISBN-0-521-08382 hard covers ISBN-0-521-29733-8 paperback. (Cambridge and London: Cambridge University Press, 1979.) £6.50 paperback.

Chemistry

- ABRAHAM, R. J. (Senior Reporter) Nuclear Magnetic Resonance vol 8. A Review of the Literature published June 1977 and May 1978. Pp. xxiv + 351. ISBN-0-85186-322-1. (London: The Chemical Society, 1979.) £28.00 \$72.00.
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History

- GILLESPIE, Neal C. Charles Darwin and the Problem of Creation. Pp.xiii + 201. ISBN-0-226-29374-2. (Chicago and London: The University of Chicago Press, 1979.) £9.90.
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General

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